INSECT CARBOHYDRATE METABOLISM: PARTIAL PURIFICATION OF INSULIN-LIKE PEPTIDES AND SOME EFFECTS OF VERTEBRATE HYPOGLYCEMIC AGENTS IN INSECTS

by

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PART I

PARTIAL PURIFICATION OF INSULIN-LIKE PEPTIDES FROM INSECTS

INTRODUCTION

This research was part of an overall study to determine how insects regulate carbohydrate metabolism. First, we wished to purify a hypotrehalosemic insulin-like factor from insects. Part I describes methods for partially purifying such a factor from the tobacco hornworm, Manduca sexta (L.) and the honeybee, Apis melifera (L.). Second, we wanted to examine effects of mammalian hypoglycemic agents on insect development. Part 2 describes the action of two sulfonylureas and a biguanide in the phytophagus insect M. sexta and the stored-product insects, the Indian meal moth, Plodia interpunctella (Hubner) and the confused flour beetle, Tribolium confusum (Jacquelin du Val). This work supports the hypothesis that the hypotrehalosemic hormone and an insulin-like peptide in insects are the same chemical species and also demonstrates that drugs which affect carbohydrate physiology in vertebrates also function in insects.

LITERATURE REVIEW

Insect Carbohydrate Utilization and Metabolic Pathways

A. Energy Storage and Utilization

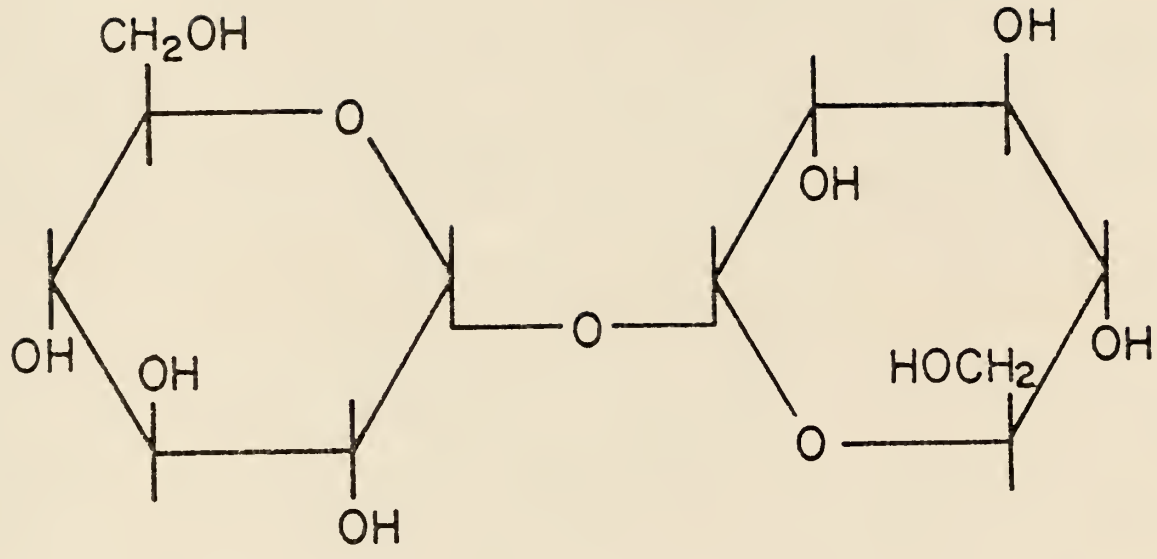
Insect and vertebrate carbohydrate metabolisms are similar. Both organisms utilize glucose as an energy source and share important metabolic pathways (57-59) like glycogen synthesis and degradation, glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway. Certain insect enzymes, e.g. glycogen synthetase (57), phosphofructokinase (58), and pyruvate kinase (59), exhibit biochemical similarities when compared to their vertebrate counterparts.

Insect and vertebrate carbohydrate metabolism also have obvious differences. In insects, the fat body carries out many functions that are divided between adipose tissue and liver in vertebrates (13). The fat body stores lipids, carbohydrates, and proteins and synthesizes many components of the hemolymph. In many insects trehalose, D-alpha-glucopyranosyl 1-(1-1)-alpha-D glucopyranoside, acts as a circulating source of stored carbohydrate, a kind of miniglycogen, (Figure 1) (12). Trehalose synthesis occurs in fat body. In the locust, Schistocerca gregaria (11), synthesis depends on the following reaction sequence:

1. Glucose-6-P + UDP-glucose --> Trehalose-6-P + UDP
2. Trehalose-6-P + H_2O --> Trehalose + Pi

The enzyme, trehalose phosphate synthetase (UDP glucose:

Figure 1. The structure of trehalose, D-alpha-glucopyranosyl 1-(1-1)-alpha-D glucopyranoside.



TREHALOSE

D-glucose-6-phosphate 1-glucosyltransferase) catalyzes reaction 1. A phosphatase (trehalose-6-phosphate phosphohydrolase) catalyses reaction 2.

After synthesis and release into the hemolymph, trehalose cannot be used for energy by the cells until it is converted to glucose. The enzyme trehalase or trehalose glucohydrolase hydrolyzes the disaccharide to release its glucose components (12,13). The reaction catalyzed by trehalase is as follows:

$$\text{Trehalose} + H_2O \rightarrow 2\ \text{D-glucose}$$

Trehalase is widely distributed in many tissues such as muscle, gut, fatbody, and hemolymph and has been found to be both soluble and particulate in nature (54-56).

B. Regulation of Carbohydrate Metabolism

Current understanding of the factors regulating concentration of blood trehalose and its metabolism is limited. However, blood trehalose homeostasis has been shown to be regulated (9,13) and appears to result from a steady state between carbohydrate input and trehalose synthesis on one hand, and sugar loss or utilization on the other. Carbohydrate input into the system is derived from both exogenous carbohydrate and gluconeogenesis. Studies on trehalose synthesis have demonstrated its control at the enzyme level. In the blowfly, Phormia regina, trehalose inhibits its own synthesis by a feedback mechanism (60). Studies of larval-cecropia trehalose phosphate synthetase

showed end product inhibition of the enzyme by trehalose and allosteric activation by glucose-6-phosphate (15). Excretion of trehalose also appears to be regulated (12,61).

Several reports have presented evidence for the presence of counter-regulatory hyper- and hypotrehalosemic hormones in insects. In 1961 Steele found a hypertrehalosemic hormone in the neurosecretory system (corpus cardiacum) of the cockroach, Periplaneta americana. Further studies characterized and confirmed the presence of this factor in the corpus cardiacum of other insects. It promoted glycogenolysis and increased both trehalose synthesis and its release into hemolymph. Its action was comparable to glucagon in vertebrates (71,72). The demonstration that purified extracts of this factor cross-reacted with vertebrate glucagon antibodies provided further evidence that the peptide was similar to glucagon (70).

In vertebrates, the antagonistic effects of the hyperglycemic hormone, glucagon, and the hypoglycemic hormone, insulin, are important determinants of blood glucose levels (62). The presence of a glucagon-like hypertrehalosemic and glycogenolytic factor in insects implied that an insulin-like hypotrehalosemic factor might also be present. In the past relatively little evidence had been obtained suggesting a hypotrehalosemic factor in insects. However, recently reports of an insulin-like

hypotrehalosemic hormone in several insect species have appeared. Seecof and Dewhurst (76) reported immunological cross reactivity with bovine insulin antibody to a factor in Drosophila melanogaster hemolymph. Whole body extracts of Drosophila were shown to produce hypoglycemia in mice by Meneses and Ortiz (84). In 1975, T. C. Norman (81) suggested that hypertrehalosemia resulting from decapitation of the blowfly, Calliphora erythrocephala, was caused by loss of a cephalic hypotrehalosemic hormone. A short time later, Tager et al. (70) showed that purified aqueous extracts of the neuroendocrine system of adult tobacco hornworm, Manduca sexta, produced hypotrehalosemia when injected into the larval form of the same species. Radioimmunoanalysis of the material revealed an immuno-reactive insulin-like component in the extract. These reports and others described in Section III strongly suggested the presence of an insulin-like peptide in insects which functions similarly to vertebrate insulin.

Insulin

A. Structure

Banting and Best first isolated the hormone insulin from dog pancreas in 1921 (32). It was not until the 1950's that its amino acid sequence was determined by Sanger and his colleagues (33). Insulin has a molecular weight in the range of 6,000 daltons (63) depending on the species. Human

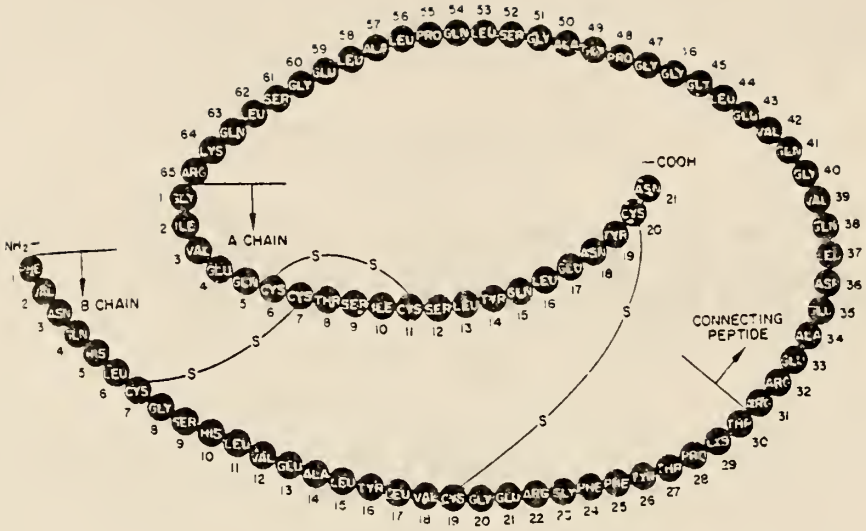
and bovine insulin have molecular weights of 5,734 daltons (129).

Insulin is composed of two polypeptide chains which are designated A and B. The primary structure of human proinsulin and insulin is shown in Figure 2. The sequences of insulin from other species are quite similar (129). In the insulin sequences from mammals, birds, and fishes which are currently known, the A chain has 21 amino acid residues and the B chain usually has 30. These chains are connected to each other by two interchain and one intrachain disulfide bond. In porcine insulin, the disulfide bonds are located between cysteine residues at position A-7 and B-7, A-20 and B-19, and A-6 and A-11 respectively. The interchain disulfide bonds must be intact for biological activity and appear to be conserved in insulins from all species studied (120).

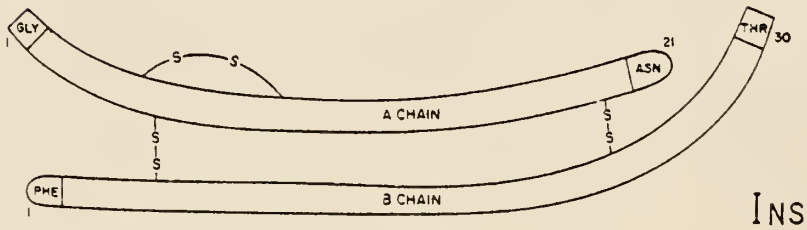
The three-dimensional structure of zinc crystalline insulin was determined by Hodgkin and her coworkers using X-ray crystallography (121,122). Hodgkin showed that the A chain of the molecule is a compact unit around which the B chain is wrapped. Insulin forms dimers and hexamers (107) and sometimes has a zinc requirement for crystallization depending on the species (64).

When considering the evolutionary origin of insulin and the possibility of insulin occurring in primitive organisms, it is interesting to note differences and similarities between hagfish and mammalian insulin. Hagfish insulin is

Figure 2. Human proinsulin and its conversion to insulin. The amino acid sequence of porcine proinsulin is shown. By proteolytic removal of four basic amino acids and the C-peptide, proinsulin is converted to insulin (adapted from Goodman and Gilman, 163).



PROINSULIN



INSULIN

considered the most ancient insulin for which detailed structural knowledge has been obtained. Although almost half of the residues in the amino acid sequence of hagfish insulin differ from those of human insulin, x-ray crystallographic studies (67) have shown the molecular backbone in hagfish insulin to be essentially identical to mammalian insulin. Because of these similarities, it is perhaps not unreasonable to expect that insulins from other primitive animals such as insects are also similar to mammalian insulin.

B. Synthesis

Steiner and associates (21,22) studied insulin biosynthesis by incorporating ^{14}C-leucine into an insulin-producing tumor. Their studies demonstrated the formation of a large precursor insulin molecule as part of the biosynthetic process. This precursor, a single polypeptide chain, was subsequently called proinsulin (Figure 2). Proteolytic cleavage of proinsulin between amino acids 30 and 31 and at the N-terminal of the A chain is required to release both insulin and a nonfunctional connecting peptide (C-peptide) from the proinsulin molecule. In many mammalian insulins a pair of basic amino acids on either side of the connecting peptide link the C-peptide to the A and B chains. Those amino acids are split out during conversion of proinsulin to insulin and are not found in insulin or the C-peptide.

The molecular weight of mammalian proinsulin ranges around 9,000 daltons (63). Hagfish proinsulin is similar in size to the proinsulins of higher species and its connecting polypeptide segment is made of similar amino acids (68).

Mammalian insulin and its precursor proinsulin share many immunologic determinants. Immunologic cross-reactivity between insulin and proinsulin of the same species has been demonstrated. However, a wide variation in the degree of such cross-reactivity has been observed (19,34-38). In general, most insulin antisera react less readily with proinsulin than with insulin. The amount of proinsulin biological activity that can be demonstrated also varies from tissue to tissue (43) but is consistently lower than that of insulin (41,42). It is likely that the proinsulin connecting segment interferes with the binding of some insulin antibodies or receptors to the insulin moiety or, perhaps, attachment of the connecting segment to the A and B chains decreases the affinity of insulin antibodies or receptors for the insulin moiety by changing its three dimensional structure.

Insulin is the first polypeptide hormone for which the mechanism of biosynthesis was elucidated (21). Insulin biosynthesis occurs in specialized endocrine, beta-cells. A preproinsulin, consisting of proinsulin with an amino terminal extension of approximately 2,500 daltons, is the first polypeptide formed in the biosynthetic pathway. It was discovered by in vitro translation of mRNA molecules

coded for producing insulin. A heterologous system lacking proteolytic enzymes was used for the translation. During *in vivo* synthesis, the amino acid terminal extension of proinsulin is rapidly cleaved off by proteolytic enzymes. Consequently, preproinsulin is usually not observed *in vivo*.

C. Action

Hormones are substances produced by specialized cells and released into the circulatory system to exert their effect on target tissues. Hormone concentrations in the blood are generally low. For example, in humans the normal insulin concentration after fasting ranges from 0.2-3 ng/ml (approximately .4-6 x 10^{13} M) (124). Most evidence indicates that insulin affects cellular functions after it binds to a cell surface receptor (16,17,18). However, the detailed molecular mechanism is unknown (51,127). A recent report (50) indicated the presence of insulin receptors in cell nuclei, but their significance in insulin action has not been ascertained.

In vertebrates insulin is an anabolic hormone and primarily affects liver, muscle, and adipose tissue. Insulin activates certain enzymes, e.g. glycogen synthetase (18) and pyruvate dehydrogenase (125), and changes membrane permeability (124). In liver, insulin increases lipid, protein, and glycogen synthesis and decreases gluconeogenesis (124). In muscle, insulin increases

glycogen synthesis, glucose uptake, amino acid uptake, and protein synthesis (124). In adipose tissue, insulin increases glucose uptake and triglyceride synthesis and decreases triglyceride breakdown by lipase (124). In addition to its anabolic effects, insulin has been shown to accelerate cell differentiation in both vertebrate and invertebrate tissue cultures (53-55).

Insulin-like Peptides in Insects

Early attempts to demonstrate a physiological response in insects to vertebrate insulin yielded unexpected results, probably because many insulin preparations used were contaminated with glucagon. No depression of blood sugar was observed in a 1924 study by Hemmingsen of the action of vertebrate insulin in the butterfly Smerinthus ocellatus (93). Although Hemmingsen recorded the amount injected into the butterfly as that of "1/10 of the rabbit dose," we have been unable to determine the actual amount. Similar results (lack of blood sugar depression) were obtained in a 1936 study (73) using the silkworm, Bombyx mori (L.) where 4 ug insulin per insect was injected. The slight increase in hemolymph sugar levels which was observed in these insects was unexpected. Krahl (84) concluded in his review that these responses were due to trace glucagon contamination of the samples of insulin employed in these early studies. Even today with more sophisticated isolation techniques,

unless extreme precaution is taken, commercial samples of insulin are usually contaminated with glucagon (124).

More recently Bhaktan and Gilbert (74) demonstrated that vertebrate insulin (20 ng insulin in 2 ml of hemolymph per 150 to 200 mg fat body) inhibited diglyceride and free fatty acid release in pupal fat body of the American silkmoth, Hyalophora cecropia. This effect was similar to the activity of insulin in vertebrates (124). In contrast, Chang (75) found that vertebrate insulin (50 ug/ml) exhibited the opposite effect in the monarch butterfly, Danaus plexippus (L.) and sweetpotato hornworm, Agrius cingulata (Fabricius). The hormone induced triglyceride, diglyceride, and free fatty acid release from larval fat body. The latter response was actually typical of vertebrate glucagon (124) and perhaps the sample of insulin used in this study was contaminated with glucagon.

In 1976, Ishay et al. (85) induced hypoglycemia in vivo in oriental hornet larvae, Vespa orientalis using bovine insulin injections (32 ug per insect). He also observed an in vitro drop in glucose levels when bovine insulin (480 to 960 ug per ml of hemolymph, a whopping high amount) was added to hemolymph which had been incubated with hornet midgut. In 1978 Norman and Duve demonstrated hypotrehalosemia (87) in the blowfly, Calliphora erythrocephala, after porcine insulin injection (8 ug per insect). The experiments by Bhaktan and Gilbert (74), Ishay et al. (85), and Norman and Duve (87) demonstrated that

vertebrate insulin can indeed affect certain insects in a manner similar to its characteristic vertebrate action.

Insulin facilitates anabolic processes in vertebrates by increasing glycogen, protein, and triglyceride synthesis *in vivo* (124). It has similar effects on vertebrate cells in culture (79,80). The results of Seecof and Dewhurst (76) demonstrate such effects on insect embryonic cells in culture. Bovine insulin added to *Drosophila* embryonic cells facilitated cellular differentiation and anabolism. The hormone (148 ng/ml) increased the number of muscle and fat-containing cells and also the total protein content of the culture by 50 percent.

Establishment of *Drosophila* cell lines in culture is also facilitated by insulin. Normally the number of cell lines obtained without insulin is very low, about one in forty. In 1976, Mosna and Barigozzi (77) were able to increase the number of cultures established by about four times (to one in ten) by adding vertebrate insulin to the culture medium. Concentrations of 74, 148, and 296 ng/ml were used and all produced about the same response. In the same year, Davis and Shearn (78) noted that mammalian insulin (16 ng per ml) enhanced growth of *Drosophila* imaginal discs in culture. Imaginal discs are undifferentiated larval and pupal cells which form wings, legs, and other organs of the adult.

The studies discussed heretofore revealed effects of vertebrate insulin in various insects or insect tissues and

suggested that insulin is active in insects. Other investigations have used bioassay and radioimmunoassay to provide additional evidence for insulin-like peptides in insects. Table 1 summarizes the results of such studies. Insulin has been quantitated in extracts of whole body of Drosophila melanogaster (84), Ceratitis capitata (98), and Apis mellifera (86); hemolymph from Drosophila melanogastor (76) and Manduca sexta (Kramer 1977, unpublished); corpus cardiacum and corpus allatum from M. sexta (70); gut tissue from Locusta migratoria (85) and several hymenopteran species (85); and royal jelly (82,83), worker jelly (82), and gut tissue (85) from Apis mellifera. Levels detected range from a ng to more than a ug per gm of tissue or ml of hemolymph.

The insulin-like peptide in royal jelly, a pharyngeal secretion of the honeybee, Apis mellifera, was first studied in 1964 (82). At that time, Dixit and Patel separated a peptide from royal jelly using paper chromatography and paper electrophoresis that had the same characteristics as bovine insulin. It produced an insulin-like effect in rat adipose tissue, increasing glucose utilization and oxidation. Kramer et al. (83) found that injection of aqueous extracts of royal jelly into M. sexta larvae caused hypotrehalosemia. Using insulin radioimmunoassay, the latter authors also demonstrated the presence of an insulin-like peptide in royal jelly that had been delipidated, acid extracted and chromatographed on

Table 1—Occurrence of insulin-like peptides in insects.

Species	Source	Assay	Quantity*	Reference	Comments
Drosophila melanogaster	Whole body	Hypoglycemia in mice	1600	(110)	Acid alcohol extract used
Ceratitis capitata	"	RIA†	0.01-0.09	(127)	"
Drosophila melanogaster	Hemolymph	RIA	16.4	(102)	No experimental detail given
Manduca sexta	"	RIA	1.0	(unpublished)	Hemolymph treated and gel filtered
"	Corpus cardiacum/ corpus allatum complexes	RIA	1 ng/complex	(96)	Gel filtered extracts used
"	"	Hypotrehalosemia in M. sexta larvae	(+)	"	"
Locusta migratoria	Gut	RIA	440	(111)	RIA performed on supernatant from homogenized tissue
Hymenopteran§ Species	"	RIA	60-1400	"	"
Apis mellifera	Adult gut	RIA	160-320	"	"
"	Larval midgut	RIA	600	"	"
"	Whole body	RIA	160	(112)	Gel filtered extract used

Table 1—continued.

Species	Source	Assay	Quantity	Reference	Comments
Apis mellifera	Royal jelly	Glucose oxidation in rat adipose tissue	240-290	(108)	Acid alcohol extract used
"	Worker jelly	"	130-240	"	"
"	Royal jelly	RIA	25	(109)	Acid extracted, delipidated, and gel filtered sample used
"	"	Hypotrehalosemia in M. sexta larvae	(+)	"	Acid alcohol extract used

*Units are ng vertebrate insulin equivalents per g tissue or ml of fluid or if not quantitated denoted as positive by (+).

†RIA is used as an abbreviation for radioimmunoassay.

δA total of ten different hymenopteran species were examined including Apis mellifera, Vespa orientalis, Vespa crabro, Paravespula vulgaris, Paravespula germanica, Dolichovespula media, Dolichovespula saxonica, Polyrachis simplex, Chalicodoma sicula, and Chrysis sp.

polyacrylamide gel. The major immunoreactive component, which was 60 percent of the total immunoreactivity assayed, had an apparent molecular weight similar to bovine insulin.

In 1975, Norman (81) suggesed that hypertrehalosemia resulting from decapitation of the blowfly, Calliphora erythrocephala, was due to a lack of hypotrehalosemic hormone of cephalic origin. The cephalic neuroendocrine gland complex, the corpus cardiacum-corpus allatum (CC-CA), was a likely source of this hormone. Other investigators have also demonstrated hypertrehalosemia in insects after selective removal of the corpus cardiacum of the blowfly, Phormia regina, (97) or of the CC-CA in the housefly, Musca domestica, (L.) (96). Partially purified immunoreactive insulin from M. sexta CC-CA complex also caused hypotrehalosemia when injected into M. sexta larvae (70). Like the findings for royal jelly (83), the elution volume of the major immunoreactive insulin component from gel filtration of extracts of the CC-CA complex was essentially the same as that of bovine insulin. This insulin like peptide may be similar to the cephalic hypotrehalosemic hormone in the blowfly originally hypothesized by Norman in 1975. Its identification adds to the increasing evidence for a functioning insulin in insects.

MATERIALS AND METHODS

Insulin Radioimmunoassay

The Insulin radioimmunoassay kit and thimerosal were purchased from ICN Medical Laboratories Inc., Portland, Oregon (ICN). The radioimmunoassay (RIA) procedure measures insulin content by comparing the competitive binding of ^{125}I labeled porcine insulin and unlabeled insulin-like peptide with an antibody to insulin, an anti-porcine insulin immunoglobulin. The assay measured amounts of porcine insulin as low as 0.5 ng.

The reagents were prepared as follows: five g of bovine serum albumin, BSA, was added to 500 ml of 0.1M sodium borate buffer and 0.5 g thimerosal mixed to form the 1 percent BSA borate solution. The final pH of the solution was 7.4. Aliquots of insulin standard solution (512 mu/ml, porcine) were mixed with 1 percent BSA borate solution to obtain 256, 128, 64, 32, 16, 8, 4, 2, and 0 uU/ml solutions of insulin for determining the standard curve. By convention 1 mg bovine or porcine insulin represented 25 units of activity (125). Two milliliters of ca 25 uCi/ml of ^{125}I-porcine insulin solution were mixed with 100 ml of a 1 percent BSA borate solution to form the tracer solution. Five milliliters of the guinea pig anti-porcine insulin serum was dissolved in 98 ml of 1 percent BSA borate solution to form the insulin first antibody solution.

Ten milliliters of rabbit anti-guinea pig serum was

dissolved in 100 ml of 1 percent BSA borate solution to form the second antibody solution.

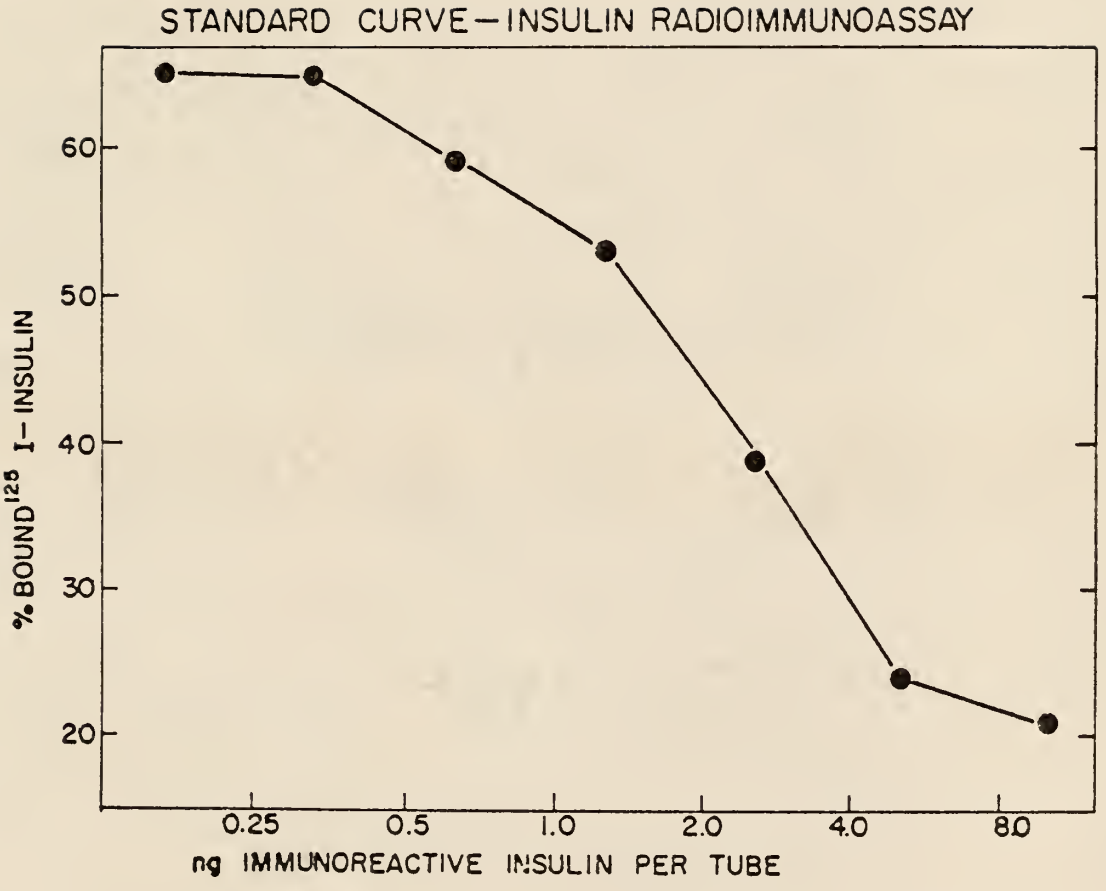
All assays were performed in duplicate. To each 13 x 100 mm test tube 0.25 ml 1 percent BSA borate solution, 0.25 ml of a solution containing insulin standard or unknown, 0.25 ml ^{125}I-insulin solution, and 0.25 ml insulin first antibody solution were added and vortexed. The tubes were then incubated at room temperature for 16 to 18 hours. Following incubation, 0.25 ml of second antibody, anti-guinea pig serum solution was added to each tube, the solution vortexed, and then incubated at room temperature for an additional four hours. After centrifugation at 5,000 rpm (3,000 x g) in a Sorval RC2B for twenty minutes, the supernatant was immediately transferred to a clean labeled tube with a Pasteur pipette. One-half ml of the supernatant was mixed with 15 ml of scintillation cocktail in a 20 ml polyethylene scintillation vial (Fisher Scientific, Pittsburgh, PA) and counted in a Searle Isocap 300 liquid scintillation counter. The scintillation cocktail was prepared by combining 1500 ml of scintillation grade toluene (Yorktown, Hackensack, New Jersey) and 1500 ml low peroxide methyl cellosolve (Mallinckrodt, St. Louis, Missouri) with 12 g Preblend 2a70 (Research Products Incorporated, Elk Grove Village Illinois, RPI). Preblend 2a70 contained 98 percent PPO (2,5-diphenyloxazole) and 2 percent bis-MSB (p-bis (0-methylstyryl)-benzene).

The fraction of labeled insulin bound by the antibody,

percent bound ^{125}I-insulin, was a function of the amount of unlabeled insulin added to the reaction mixture. Assays were performed to determine the cpm in an aliquot of the supernatant of the sample or standard and the cpm in an aliquot from a sample to which buffer was substituted for antibody and unlabeled insulin solutions. The radioactivity from the tube to which no antibody was added was used as a measure of the total ^{125}I-insulin added.

In general, the technique of radioimmunoassay depends on binding of isotopically labeled antigen by an antigen-specific antibody which is competitively inhibited by addition of unlabeled antigen. The fraction of radioactive antigen bound by the antibody is a quantitative function of the amount of unlabeled antigen added to the reaction mixture when the concentration of the antibody is held constant. When the antibody-bound antigen is separated from unbound or free antigen by precipitation with second antibody directed against first antibody, the fraction of labeled antigen bound to antibody was determined by counting supernatant. A plot of the percent antibody bound-labeled antigen against the quantity of unlabeled standard antigen added to the incubation mixtures produces an exponential curve. A more linear relationship over the useful range of the assay is obtained if the concentration of standard is plotted logarithmically. A semilogarithmic plot of a standard curve from the ICN radioimmunoassay system used in these studies is shown in Figure 3. The percent

Figure 3. Displacement curve for the ICN insulin radioimmunoassay. The percent bound ^{125}I-insulin has been plotted vs. the logarithm of the concentration of insulin for each standard solution.


STANDARD CURVE—INSULIN RADIOIMMUNOASSAY
% BOUND ^{125} I-INSULIN
60
50
40
30
20
0.25
0.5
1.0
2.0
4.0
8.0
ng IMMUNOREACTIVE INSULIN PER TUBE

^{125}I-insulin binding for each unknown sample was fitted on a standard curve to quantitate the amount of immunoreactive insulin in an unknown sample.

The Anti-Insulin Sepharose Column

A. Preparation and Characterization

Two milliliters of goat anti-porcine insulin serum (RPI) stored at -70°C were thawed and transferred from the shipping vial to a centrifuge tube followed by a 2 ml distilled water rinse. The anti-insulin immunoglobulin fraction was precipitated by addition of 0.72 g of Na_2SO_4 to the solution (18 percent salt). The solution was centrifuged at 5,000 rpm (3,000 x g) for five minutes and the supernatant decanted away. The precipitate was dissolved in distilled water and dialyzed against 0.14 M NaCl for 24 hours 4°C. The cellulose nitrate dialysis tubing (average radius permeability = 24 angstroms, VWR Scientific, Kansas City, Kansas) was prepared by boiling in 1 mM ethylenediamine-tetraacetic acid (EDTA) for one half hour and then in 1 mM sodium bicarbonate for one half hour. The tubing was then rinsed well and stored in distilled water at 4°C until use. After dialysis the total amount of immunoglobulin in the dialysate was determined to be 14 mg using E_{280} = 1.5 ml cm/mg (112).

A 2.7 gm portion of CNBr- activated Sepharose 4B (Pharmacia, Piscataway, New Jersey) was swelled with 540 ml

1 mM HCl solution for fifteen minutes, washed and filtered dry using a sintered glass filter. Then dialyzed immunoglobulin was immediately added to the beads in 15 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 percent NaCl pH 8.0) and stirred gently for three hours. After filtering to dryness the beads were then washed twice with coupling buffer and finally with a solution of 1 M ethanolamine, 0.1 M $NaHCO_3$, 0.5 percent NaCl pH 8.0 for at least four hours to react any left over electrophilic sites on the beads. Noncovalently adsorbed proteins were removed by first washing with 0.1 M NaOAc, 1 M NaCl, pH 4.0 buffer and then with a 0.1 N $Na_2B_2O_4$, 1 M NaCl, pH = 8. The insulin immunoglobulin coupled to Sepharose 4B (abreviated AIG-S) was stored at 4^oC in 20 ml of 0.1 M $Na_2B_2O_4$, 1 M NaCl, pH 8.0 buffer containing 1 percent gentamicin sulfate (Schering, New Jersey) as a preservative. The amount of immunoglobulin coupled to the column was determined by change in absorbance at 280 nm of the coupling buffer and immunoglobulin solution before and after the coupling procedure. Ninety-seven percent of the immunoglobulin was coupled to the column.

The AIG-S was packed in a glass column filled with a 0.1 percent BSA (Sigma, St. Louis, Missouri or ICN), 0.01 M Tris-HCl buffer, pH 8.2 (Tris-BSA buffer). Columns were poured to the dimensions of 1.0 x 2.0 cm or 1.0 x 6.0 cm. A control column (1.0 x 2.0 cm) of uncoupled Sepharose 4B-Cl was prepared in a similar manner.

Modifications of procedures reported by Akanuma et al. (111) were used for characterizing the coupled antibody column. ^{125}I-Insulin (0.68 uCi/ml, 7.2 ng/ml ICN, 1 to 3 ml) in a 1 percent BSA borate solution (0.1 M, pH 7.4) was applied to the small column and allowed to equilibrate. Column effluent was collected in ca 1.0 ml fractions and the amount of radioactivity of each fraction was determined. The column was washed with Tris-BSA buffer (20 ml, pH 8.2) until no radioactivity was detected in the effluent, then adsorbed radioactive peptides were eluted with 1 M acetic acid (20 ml). When no radioactivity could be detected in the acid effluent, the column was reequilibrated to pH 8.2 with Tris-BSA buffer (12 ml).

^{125}I-Gastrin with a specific activity of ca 700 uCi/ug was diluted to ca 70 uCi/ml with a 0.02 M, Tris buffer pH 8.0. One half ml was equilibrated with the column and binding was determined as described above.

A twenty-five ml of radioactive insulin solution (106 ng bovine insulin (Sigma)/ml, 7.2 ng ^{125}I-insulin (ICN)/ml and 1.2 x 10^3 cpm/ml) was applied to the insulin antibody column until it was saturated. When radioactivity of the fractions had reached a plateau value, it was assumed that maximum insulin binding capacity of the column had been reached. The column was then washed with Tris-BSA buffer (20 ml) until no radioactivity was detected in the effluent. Bound insulin was eluted from the column with 1 M acetic acid (20 ml). The column capacity was calculated from the

total cpm eluted by the acid buffer and the specific activity of labeled solution.

Manduca sexta Insulin-Like Peptides: Separation from Hemolymph

M. sexta hemolymph was obtained from fifth stadium larvae by cutting off and bleeding through the abdominal horn and then lyophilized.

A gram of freeze dried hemolymph (equivalent of about 20 ml fresh hemolymph) was extracted with 10 ml of 3 M acetic acid. Six milliliters of Tris-BSA buffer pH 8.2 was added to the lyophilized acid extract (440 mg) and 6 N sodium hydroxide was used to adjust the pH to 8.2. The suspension was centrifuged at 5,000 rpm (3,000 x g) in Sorval RC2B centrifuge for twenty minutes. The supernatant was decanted and saved. Another 6 ml of Tris-BSA buffer was mixed with the precipitate and the centrifugation step repeated. The two supernatants were combined and passed through the large insulin antibody column. The column was then washed with 12 ml Tris-BSA buffer, followed by 17 ml of 1 M acetic acid. One ml fractions of the acetic acid eluant were collected. A 0.2 ml aliquot was removed from each fraction, lyophilized and subjected to insulin radioimmunoassay.

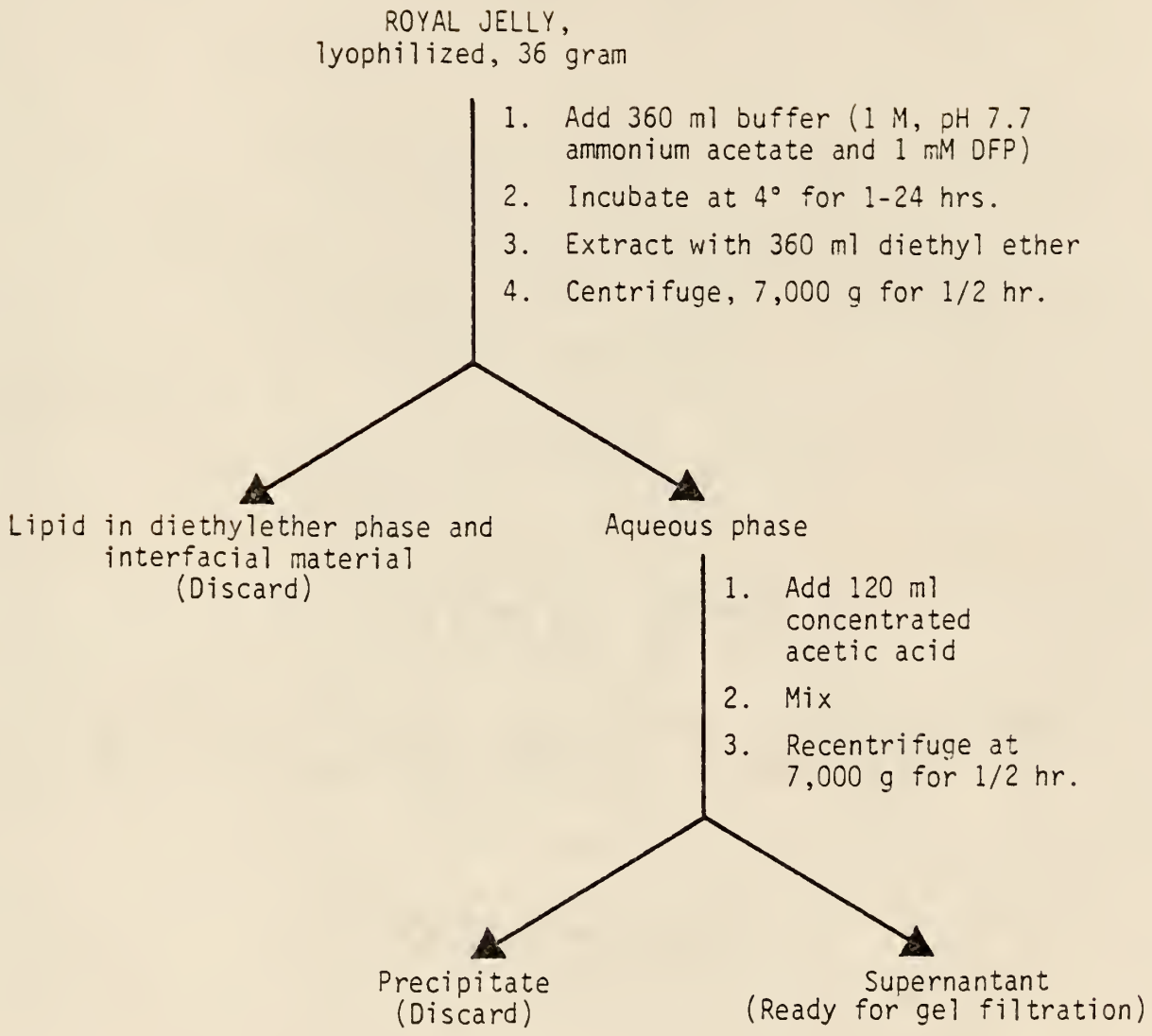
Apis mellifera Insulin-Like Peptides: Separation from Royal Jelly

A. Extraction of Royal Jelly

Royal jelly from the honeybee, *Apis mellifera*, was obtained from Dr. Nevin Weaver of the Dept. of Biology, University of Massachusetts, Boston, Massachusetts and from Formosa Joy Enterprises Co., LTD, Taiwan. The royal jelly from Dr. Weaver had been collected in 1964, immediately freeze dried, and stored under nitrogen in the deep freeze. It was used for preliminary studies. The native royal jelly obtained from Formosa Joy Enterprises was probably collected in 1977 and sent to Manhattan in a frozen state. It was then stored at -70°C until lyophilized and subsequently stored -18°C until used. Approximately 0.4 g of lyophilized royal jelly was the equivalent of 1 g of native royal jelly.

The following procedure was routinely employed to render the proteinaceous components of lyophilized royal jelly suitable for chromatographic analysis (Figure 4). The polypeptide components from 36 g of freeze dried royal jelly were solubilized in 360 ml of a pH 7.7, 1 mM ammonium acetate and 1 mM disopropyl fluorophosphate (DFP) solution. The resulting solution was incubated at 4°C for at least one hour. The sample was partially dilipidated by extraction with 360 ml of diethyl ether. The biphasic mixture was then centrifuged 7000 x g for one half hour, after which the upper ether layer and interfacial material were discarded. The remaining aqueous phase consisted of both soluble and

Figure 4. Scheme for the extraction and delipidation of royal jelly.


ROYAL JELLY,
lyophilized, 36 gram
1. Add 360 ml buffer (1 M, pH 7.7 ammonium acetate and 1 mM DFP)
2. Incubate at 4° for 1-24 hrs.
3. Extract with 360 ml diethyl ether
4. Centrifuge, 7,000 g for 1/2 hr.
Lipid in diethylether phase and interfacial material (Discard)
Aqueous phase
1. Add 120 ml concentrated acetic acid
2. Mix
3. Recentrifuge at 7,000 g for 1/2 hr.
Precipitate (Discard)
Supernantant (Ready for gel filtration)

insoluble material. Most of the insoluble material was solubilized by acidifying the suspension to a final concentration of 3.5 M acetic acid. The solution was recentrifuged at 7,000 x g for one-half hour, the remaining precipitate was discarded, and the supernatant was used for gel filtration or stored in the freezer at -18°C.

B. Gel Filtration

Porous polyacrylamide beads (100 to 200 mesh), Bio-Gel P-10, were obtained from Bio-Rad laboratories, Richmond California. The beads had a fractionation range of 1,500 to 20,000 daltons. Approximately 40 g of dry Bio-Gel P-10 were added to 800 ml of 3 M acetic acid. The gel was allowed to hydrate for more than four hours at room temperature. Then half the supernatant was decanted and the slurry was deaerated by application of a partial vacuum. A 2.5 x 75 cm column of Bio-Gel P-10 was prepared and equilibrated with 3 M acetic acid at ca 25 ml per hour overnight.

A four ml solution containing approximately 4 mg/ml of bovine serum albumin (Sigma); 2 mg/ml bovine insulin (Sigma); and 30,000 cpm/ml ^{14}C leucine (324 mCi/mM) was applied to the column, eluted with 3 M acetic acid and collected in a series of 130 tubes of 2.3 ml increments. Relative amounts of protein in each increment of eluent were estimated from its absorbance at 280 nm in a Cary 118C spectrophotometer. The amount of radioactivity in each sample was determined by liquid scintillation counting of a

0.25 ml sample.

Five ml of centrifuged acid extract of royal jelly (0.5 gm of lyophilized or 1.2 gm native royal jelly) was chromatographed on the Bio-Gel P-10 column. Fractions were collected in 3.2 ml increments. Relative protein concentrations were determined by absorbance of 280 nm in a Cary 118C spectrophotometer. The samples were pooled for radioimmunoassay into batches of 15 tubes each, beginning with tube number 3 and lyophilized.

C. Radioimmunoassay

The pooled and lyophilized samples from the Bio-Gel P-10 Chromatography were dissolved in water or 0.1 M, ammonium bicarbonate, pH 8.0. Because residual acid from the column buffer was present, the pH of the solution was adjusted to 7.4 using 10 percent ammonium hydroxide and the solution was lyophilized again. These steps (dissolving the sample, adjusting the pH, and lyophilizing) were repeated a second time to ensure that all residual acid was volatilized. Then the lyophilized samples were dissolved in 2.5 ml of 10 mM phosphate buffer pH 7.4 containing 1 percent BSA (ICN). If the pH of the samples was below 7.2, it was adjusted with small quantities of 10 percent ammonium hydroxide to pH 7.4. One ml of the resulting solution was transferred to each of two tubes and lyophilized. Samples were redissolved in 0.25 ml distilled water for insulin radioimmunoassay.

D. Affinity Chromatography

Sample pools that contained insulin-like immunoreactivity were subjected to affinity chromatography on the large antibody column as follows: pools from a series of 16 to 18 columns of gel filtration material were combined and lyophilized. Material from each of the consolidated pools was weighed and extracted in Tris-BSA buffer two times with a volume of buffer which yielded an initial concentration of 200 mg/ml. The pH was adjusted to 8.2 with 6 N sodium hydroxide before centrifuging the solution at 5,000 rpm (3,000 x g) for twenty minutes. The supernatants were decanted, pooled (final concentration 100 mg/ml) and subjected to affinity chromatography. The column was then washed with about 15 ml of Tris-BSA buffer. The effluent from the column was collected and assayed to determine unadsorbed immunoreactive insulin. Then the column was washed with another 20 to 30 ml of a pH 8.2 M Tris buffer (no BSA). Adsorbed immunoreactive insulin was eluted from the column with 1 M acetic acid and the fractions were subjected to insulin radioammunoassay.

RESULTS

Sources of Insect Insulin-like Polypeptides

Two tissues were used as starting material for isolation of insulin-like polypeptides from insects. Royal jelly from the honeybee, Apis mellifera, was utilized because Dixit and Patel reported that it contained a peptide (82) which elicited an insulin-like effect (glucose oxidation) in vertebrate adipose tissue. Also Kramer et al. (83) described a peptide from royal jelly that induced hypotrehalosemia in the tobacco hornworm and cross-reacted with porcine insulin antiserum obtained from rabbits.

The other tissue used as a source of an insulin-like polypeptide was hemolymph from Manduca sexta larvae. Since hormones by classical definition are transported in blood and Tager et al. (70) described an insulin-like peptide in certain neuroendocrine glands of M. sexta, hornworm hemolymph seemed a logical starting material.

Insect Insulin Assay System: Problems Encountered with Radioimmunoassay

Bioassay and radioimmunoassay are the two methods most used to detect insulin-like peptides. Bioassays are more detailed, time consuming, not as reproducible, and usually require a larger sample size than radioimmunoassay (RIA) to produce a statistically sound assay. In general a radioimmunoassay is cheaper, technically simpler to conduct

and more sensitive and reproducible than bioassay procedures. Radioimmunoassay was the method used in this study of insect tissues.

Two different commercial insulin radioimmunoassay systems were tried in these experiments. We first attempted to use an inexpensive Research Products International (RPI) component oriented system. RPI supplied guinea pig anti-bovine insulin antibody serum, goat anti-guinea pig serum, and guinea pig carrier serum. The RPI kit did not provide buffer, insulin standards, or iodinated insulin.

Several problems were encountered with the use of the RPI system. Optimal sensitivity of a radioimmunoassay is obtained when approximately 50 percent of labeled hormone is bound by antibody in the absence of unlabeled hormone (128). With the RPI system, less than 5 percent of the tracer ^{125}I-porcine insulin (ICN) was bound by the anti-bovine insulin antibody in the absence of standard bovine insulin. An investigation was made to determine if the absence of binding resulted from a lack of cross-reaction of the porcine insulin with the anti-bovine insulin antibody. Since porcine insulin differs from the bovine counterpart by only two amino acids, (129) and since most antibodies to porcine or bovine insulin are 98 percent cross-reactive (130), it appeared that decreased cross-reactivity was not the cause of our problem. Attempts to use a ^{125}I-bovine insulin tracer were also unsuccessful. We tried using a homemade bovine insulin tracer (first with the original RPI

system, second with the same system, but using a second shipment of completely new antibodies which were guaranteed functional by RPI, and third with the RPI system, new first antibody, and ethylene glycol 6000 as the complex precipitating agent). No tracer binding was observed in any of these experiments. These results indicated that absence of binding was not due to lack of cross-reactivity or to defective antibodies.

It was suspected that the ^{125}I-peptide did not bind because of some other component present in the buffer system. This apparently was the problem. A binding assay was conducted with components which had been successfully utilized in the RPI quality control lab. These components--antibodies, ^{125}I-bovine insulin, and phosphate buffered saline buffer (BSA-PBS)--produced about 30 percent ^{125}I insulin binding. This binding did not occur, however, when the homemade BSA-PBS buffer was used.

Thus, there were one or more components in the homemade BSA-PBS buffer that prevented antigen antibody binding in the RPI radioimmunoassay. Research Products personnel suggested that some commercial forms of BSA were contaminated with an "insulinase." We had used BSA fraction V from Sigma in our buffer. When the binding assay was performed again, but this time utilizing a BSA-PBS buffer made with purified BSA from RPI, about 50 percent of the insulin binding capacity was restored. This result implied both the Sigma BSA and other components of the BSA-PBS

buffer prevented binding. Other components of the BSA-PBS buffer system included salts, such as NaCl, Na_3PO_4, NaOH, and EDTA. It is unlikely that interference resulted from the reagent grade chemicals used here. RPI representatives suggested that insufficiently purified water may also have inhibited insulin binding. However, use of distilled water gave satifactory results with other radioimmunoassays performed in this lab. We did not investigate the RPI radioimmunoassay system further because another commercially available RIA system for insulin was found that was adequate for our needs. The kit from ICN Medical Laboratories Inc. provided necessary reagents. It produced 60 to 65 percent ^{125}I insulin binding in the absence of unlabeled insulin, and detected as little as 500 pg mammalian insulin.

Extraction of Insulin-like Peptides from Royal Jelly

The best extraction technique is one that results in the selective solubilization of the desired peptide in a protective solvent. This condition is often difficult to achieve because numerous other peptides with similar solubility properties are present. It is important to extract under conditions that inactivate substances which may damage the peptide or interfere with its assay. Vertebrate insulin, like most peptides, is highly water soluble and an aqueous solution with low salt concentration can be used for its extraction. However, there is danger to

the integrity of the insulin molecule from coextracted proteolytic enzymes. This can be minimized by using proteolytic enzyme inhibitors, extremes of pH, or heat treatments as a part of the extraction procedure. Protection of the insulin molecule is possible because it is stable under these conditions and proteolytic enzymes are not.

Extraction methods for the insect insulin-like peptides in royal jelly were developed to solubilize the peptides, to remove substances which might interfere with the assay, and to inactivate any proteolytic enzymes which may be present. Acidification was used to solubilize the peptide components of the sample. The solubilization and acidification of the sample were performed at 4°C and in the presence of disopropyl-fluoro-phosphate (DFP, a serine hydrolase inhibitor) to inactivate proteases which may have been present. Royal jelly differs from ordinary sources of insulin-like peptides because it has a relatively high lipid content (about 16 percent on a dry weight basis (140)). This lipid appeared to interfere with the radioimmunoassay. The royal jelly was extracted with diethyl ether to remove interfering lipids from the sample.

Gel Permeation Chromatography of Royal Jelly Immunoreactive Insulin

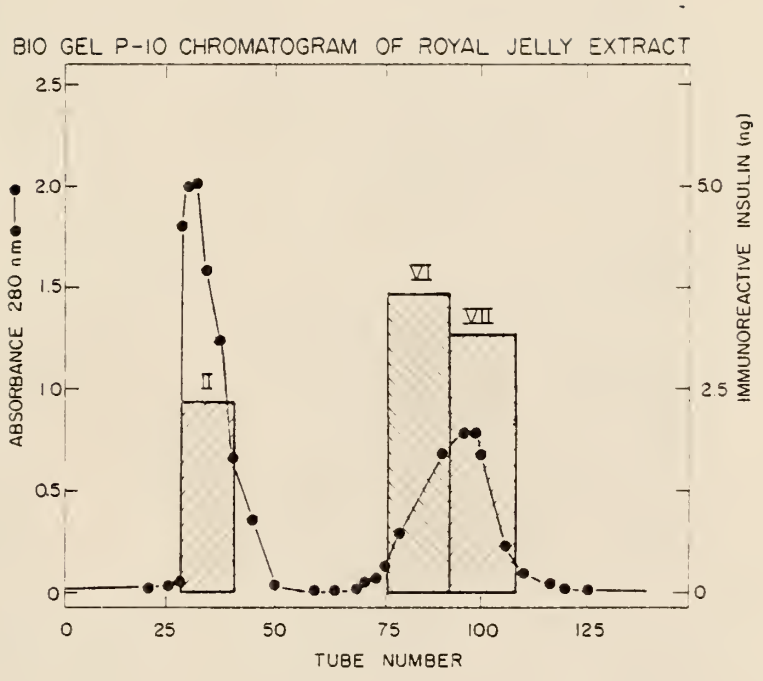
The acid extract of royal jelly containing the equivalent of 1.2 g native royal jelly, was chromatographed

on a Biogel P-10 column in 3 M acetic acid. In Figure 5, the elution volumes of BSA, bovine insulin, and ^{14}C-leucine markers are shown. BSA with a molecular weight of 6.7×10^4 daltons was used to mark the void volume of the column (109 ml); bovine insulin, the insulin-like peptide volume (243 ml); and ^{14}C-leucine, the inclusion volume of the column (314 ml). Figure 5 also shows the elution profile of royal jelly as measured by the absorbance at 280 nm of the effluent. Two peaks were observed, one at the void volume of the column and the other right before the inclusion volume.

In order to minimize the number of assays performed and increase the amount of immunoreactive insulin (IRI) in an individual assay, pools of 15 tubes each beginning with fraction 3 were collected from the column and lyophilized. As shown in Figure 5, IRI was found in three of the eight pools. Amounts detected were 2.4 ng in pool II (void volume), 3.6 ng in VI (elution volume of bovine insulin) and 3.2 ng in VII (inclusion volume). IRI was not detected in the remaining pools. The total IRI assayed in the eluant was 9.2 ng porcine insulin equivalents or about 7.7×10^{-7} percent of the native royal jelly.

The pool II immunoreactive components have a relatively large molecular weight, $\geq$ 10,000 daltons. This may be an aggregate of immunoreactive insulin or some precursor form similar to vertebrate proinsulin. The former is unlikely because the acidic solvent used does not favor association.

Figure 5. Bio-Gel P-10 chromatogram of royal jelly extract. The profile of absorbance at 280 nm (●———●) obtained after gel filtration of an acid extract of royal jelly from Apis mellifera has been superimposed on a bar graph which indicates positions and quantities of immunoreactive insulin found after assay of pools of the column effluent. The arrows mark the elution position of the standards from left to right: BSA, bovine insulin, ^{14}C-leucine.


BIO GEL P-10 CHROMATOGRAM OF ROYAL JELLY EXTRACT
ABSORBANCE 280 nm
IMMUNOREACTIVE INSULIN (ng)
TUBE NUMBER
II
VI
VII
0
0.5
1.0
1.5
2.0
2.5
2.5
5.0
25
50
75
100
125

The IRI in pool VI may correspond to the actual insect insulin hormone, because it is similar in size to bovine insulin. Some of the IRI found in pool VI and all of the IRI found in pool VII is not insulin-like material as will be explained below. It is actually composed of low molecular weight substances that give a false positive response in the radioimmunoassay.

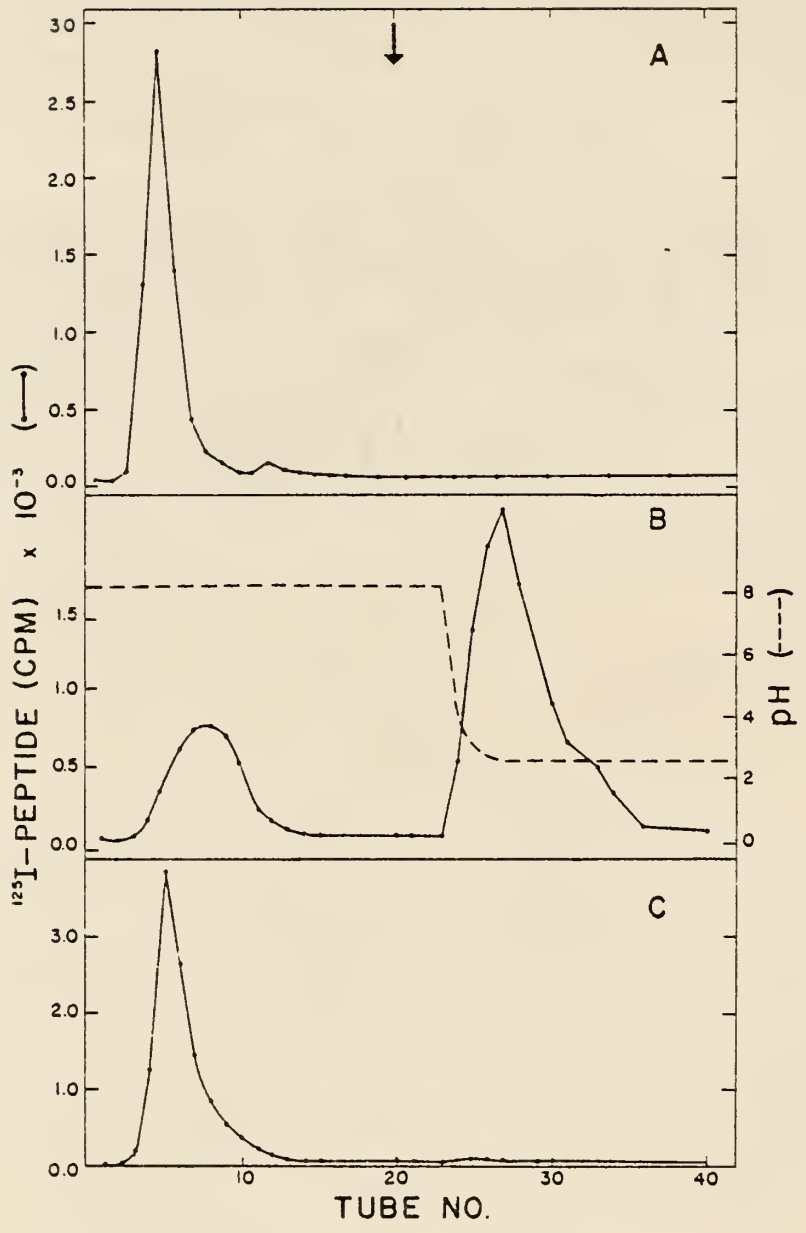
Affinity Chromatography of Immunoreactive Insulin from A. mellifera Royal Jelly and M. sexta Hemolymph

Affinity chromatography was selected as the third step in the isolation procedure for immunoreactive insulin-like peptides in royal jelly. An affinity column with anti-porcine insulin immunoglobulin coupled to agarose was prepared as described in Materials and Methods.

A. Characterization of the Insulin Antibody Column

First, the possibility of nonspecific binding of insulin to agarose (sepharose) was studied (Figure 6). In each study, after a sample was applied to the column and the column washed with pH 8.2 buffer, adsorbed material was eluted with 1 M acetic acid. In Figure 6 the arrow indicates where the acid elutant was added to the column (fraction 20). When a standard solution of ^{125}I-bovine insulin was subjected to chromatography on a agarose column, no radioactivity was adsorbed. All of the peptide eluted in fractions 4-8. Thus, no interaction between insulin and

Figure 6. Binding of ^{125}I-peptides to agarose and anti-insulin globulin-coupled agarose. Sample was applied in pH 8.2 buffer and adsorbed material eluted with 1 M acetic acid beginning with fraction 20. A. Application of ^{125}I-insulin, 7800 cpm to agarose. B. Application of ^{125}I-insulin, 24,000 cpm to antibody column. C. Application of ^{125}I-gastrin, 15,000 cpm to the antibody column.


A
B
C
^{125}I-PEPTIDE (CPM) x 10^{-3} (——)
pH (- - -)
TUBE NO.
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
2
4
6
8
10
20
30
40

agarose occurred under the conditions of sample application.

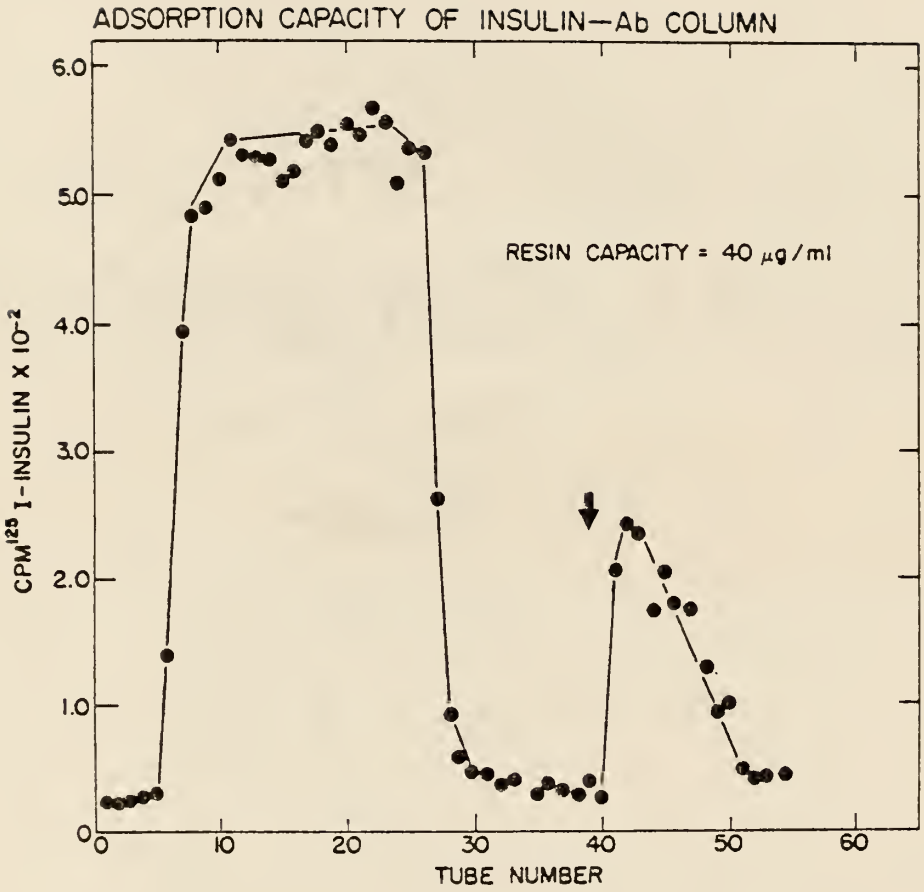
Second, binding of insulin to the immunoglobulin coupled agarose was measured (Figure 6B). Twenty-two percent of the radioactivity applied did not bind to the column and eluted in the pH 8.2 wash buffer. This material may have been radiation damaged peptide or free radioactive iodide derived from decomposition of iodinated peptides. Adsorbed insulin eluted as a sharp peak in fractions 23-32 as the pH of the eluant decreased from 4.0 to 2.5. This peak was 58 percent of the total counts added. Nineteen percent of total counts added was unaccounted for. This radioactivity may have been bound irreversibly to the column material or have been lost in the background counts of the eluant. Akanamu et al. (111) experienced a similar loss of radioactivity (8 percent) with their column.

To determine reproducibility of insulin binding to the immunoglobulin coupled agarose, the above experiment was repeated twice using the same experimental conditions as above but only one-third the quantity of tracer. In each of the three trials, 20.6 $\pm$ 1.1 percent of the ^{125}I-material was recovered in the wash buffer and 57.8 $\pm$ 0.9 percent was eluted with 1 M acetic acid. Approximately 20 percent of the radioactivity was always unaccounted for. From the reproducibility of these results, it was concluded that repeated acetic acid treatments did not change insulin binding properties of the antibody column.

The specificity of the insulin antibody coupled to agarose was examined by determining whether another peptide hormone would adsorb to the column. For this ^{125}I-gastrin was used (Figure 6C). No significant amount of gastrin was adsorbed by the insulin antibody column. All of the radioactivity eluted in fractions 4-11.

Another characteristic of the column determined was binding capacity. A radioactive insulin solution (106 ug insulin and 1200 cpm per ml solution) was applied to the affinity column until a plateau of radioactivity was achieved (Figure 7). The column then was washed with pH 8.2 buffer until no significant radioactivity was recovered in the effluent. Finally, one molar acetic acid was used to recover the adsorbed insulin from the column. The total binding capacity of the column was calculated from the amount of radioactivity deadsorbed. The 1 x 6 cm column bound 241 ug insulin. This is the equivalent of 23 ug of insulin bound per mg of column bound immunoglobulin, 40 ug per ml of packed resin or about one mole insulin bound per mole IgG. Since two molecules of antigen bind one molecule of antibody, approximately 1/2 of the IgG preparation is composed of insulin specific binding protein. The results of these experiments supported the expectation that the affinity column would function in the manner of a specific antigen-antibody reaction, and selectively bind insulin.

Figure 7. Adsorption capacity of the insulin antibody column. Twenty-five ml of a 106 ug/ml solution of ^{125}I-insulin (1200 cpm/ml) was applied to a 1 x 6 cm column in pH 8.2 buffer. Adsorbed radioactivity was eluted with 1 M acetic acid (arrow).


ADSORPTION CAPACITY OF INSULIN—Ab COLUMN
RESIN CAPACITY = 40 μg/ml
CPM 125 I-INSULIN X 10-2
TUBE NUMBER
0
1.0
2.0
3.0
4.0
5.0
6.0
0
10
20
30
40
50
60

B. Separation of Immunoreactive Insulin from Royal Jelly and Hemolymph

After establishing that affinity column had the proper specificity for insulin and enough capacity to handle our tissue preparations, we next attempted to fractionate insect insulin. The samples used were the Bio-Gel pools II, VI, and VII and also an acid extract of hemolymph from M. sexta. These materials were lyophilized and dissolved in pH 8.2 buffer at a concentration of 100 mg/ml before application to the column.

The immunoreactive insulin from royal jelly in pool II was adsorbed nearly completely by the RPI antibody column and was recovered in 70 percent yield. This immunoreactivity was composed of peptides with molecular weights greater than 10,000 daltons and is termed high molecular weight IRI for purposes of discussion. Not all of the immunoreactive insulin from the royal jelly pools VI and VII adsorbed to the affinity column. In fact no adsorption was observed in pool VII. The pool VI immunoreactive insulin had a molecular weight similar to or less than vertebrate insulin. It contained both true immunoreactive insulin (33 ng) which was adsorbed by the column antibody and also some component(s) that yielded a falsely positive response in the batch assay. Pools VI and VII eluted just before and at the inclusion volume respectively. The nonadsorbed immunoreactive insulin in pools VI and VII was probably low molecular weight material which inhibited tracer binding in the batch RIA assay.

Yields of adsorbed IRI from royal jelly were 1.4, 1.5, and 0 ng per gram native jelly for pools II, VI, and VII respectively or a total of 2.9 ng/g. Thus, the percentage of dry weight of adsorbed IRI in native royal jelly was 2.9×10^{-7} percent.

We also used the affinity column to separate IRI from M. sexta hemolymph. An acid extract from 1 g freeze dried hemolymph was applied to the column and apparent immunoreactive insulin was eluted both in the pH 8.2 buffer (48 ng) and in the acid buffer (102 ng). A total of 150 ng equivalents of IRI was obtained by ICN assay. Like the nonadsorbing material identified in royal jelly, the 48 ng of nonadsorbed apparent IRI in hemolymph was probably not peptide material. The 102 ng adsorbed by the column antibody was 1×10^{-5} percent of the lyophilized hemolymph. As observed with pool VI of royal jelly, the adsorbed reactivity was composed of peptide material with an apparent molecular weight of 5000 daltons (Kramer, K. J. and Childs, C. N., unpublished). Assuming this size, about 10^{-9} M peptide was estimated to be present in the hemolymph. However, this level is probably a low estimate since the extraction procedure may not be completely efficient and also since the affinity of the antibody for the insect peptide is, in all probability, much less than that for the vertebrate peptide.

DISCUSSION

Radioimmunoassay and Separation of Insulin-like Peptides from Insects

Radioimmunoassay of peptide levels in biological tissues is not a straightforward procedure. The results of this study and other investigations have demonstrated that such an assay is not reliable unless certain precautions are taken. For example, initial attempts in this study to determine insulin concentration failed because of the presence of interfering substances in the assay buffer. Further studies showed that valid estimates of insect insulin could be obtained only in partially purified samples and even these had to be corrected for interference from substances that disrupt tracer peptide binding in the batch assay.

Ishay et al. (85) and Kramer (unpublished) experienced problems with radioassay of insect insulin. They could not detect immunoreactive insulin in native royal jelly. Only after subjecting the jelly to acid extraction, delipidation and gel filtration was Kramer et al. (83) able to detect 25 ng insulin-like peptides per gram of native material. In the present study, the Kramer et al. (83) procedure was repeated, but only 7.7 ng immunoreactive insulin was detected--about one-third the amount originally reported. Since the fractionation procedures employed were the same in both studies, the differences in the levels of apparent

immunoreactive insulin could be attributed to different sample sources, handling conditions and/or immunoassays. Kramer et al. (83) obtained their royal jelly from U. S. suppliers and used a rabbit anti-bovine insulin radioimmunoassay system, while this study used royal jelly from a company in Taiwan and a guinea pig anti-porcine insulin system. In the present study, it was also determined that estimates of insect insulin obtained from these partially purified royal jelly samples had to be corrected for the presence of false positive IRI material. The use of a fourth clean-up procedure, affinity chromatography, demonstrated that only 40 percent or 2.9 ng of the 7.7 ng of apparent immunoreactive insulin detected after gel filtration was true immunoreactive insulin. The advantage of gel filtration followed by affinity chromatography was that it allowed discrimination of true immunoreactive species according to molecular size. Royal jelly contained both an immunoreactive insulin similar in size to vertebrate insulin and a larger immunoreactive species, perhaps chemically similar to vertebrate proinsulin. About half of this amount was similar in size to vertebrate insulin, while the remaining IRI was large molecular weight IRI.

Preliminary experiments with untreated M. sexta hemolymph showed that some peptide clean-up procedure was necessary before a valid radioimmunoassay could be conducted. Based on previous experience with royal jelly

IRI, it was decided to utilize affinity chromatography of a hemolymph acid extract for such a clean-up. This worked quite well and hemolymph was found to contain approximately 100 ng IRI per g dry weight. The heterogeneity of this material was not determined in this study. Recently, Kramer et al. (unpublished) have found that 70 percent of the IRI in hornworm hemolymph is peptide material with an apparent molecular weight of 5000 daltons.

These studies using insect hemolymph and royal jelly describe the first attempt to isolate insulin from insects using affinity chromatography. Traditional techniques, such as ion-exchange chromatography or isoelectric precipitation, were not used because of the low insulin content of our starting materials. Affinity chromatography not only fractionated our sample but also concentrated it to a level where a radioimmunoassay could be reliably utilized.

The quantities of insect IRI fractionated here were quite small because of the limited amount of tissue used. If larger quantities of tissue become available, procedures used here can be scaled up and used with other purification steps, such as paper chromatography (95), polyacrylamide gel electrophoresis, or ion-exchange chromatography (132) to yield enough purified IRI for further chemical characterization such as amino acid and sequence analyses. If the amino acid sequence of insect IRI was found to compare favorably with that of vertebrate insulin, then this result would constitute the best evidence for the presence

of insulin in insects. Also purified material could be used in physiological studies to determine effects on carbohydrate metabolism. In addition it could be used as an immunogen for insect insulin specific antibody production. Such antibodies could be used to develop a more specific and sensitive radioimmunoassay for insect insulin and also to prepare a more selective affinity chromatographic column.

Evolutionary Aspects of an Insulin-like Peptide in Invertebrates

Insects are a major class of the phylum Arthropoda. Arthropods are among the most advanced of all invertebrates (102) and have many chemical characteristics similar to vertebrates. Insulin-like peptides have been identified previously by immunological and biological assays in three orders of insecta: Hymenoptera (82,83), Lepidoptera (70), and Diptera (76,81,84,98). The results of this investigation have confirmed the presence of immunoreactive insulin in royal jelly from A. mellifera and have demonstrated for the first time an insulin-like peptide in hemolymph from M. sexta.

The level of IRI in royal jelly was 2.9 ng/g native tissue. Dixit and Patel (82) reported ca 70 times that amount based on biological activity. The different results may be explained by the fact that we used a heterologous radioimmunoassay system (one that was developed using one animal species and utilized in another) to identify in

insects a peptide previously characterized in vertebrates and Dixit and Patel used bioassay. The amino acid sequences of peptides from different species have diverged during the course of evolution. This divergence has frequently occurred through single base mutations, and has generally been conservative, resulting in little alteration in biological function of many of the peptides. Species differences in a hormone are, therefore, likely to occur in regions of the molecule that are not directly involved in its biological action and decreases in immunological cross-reactivity are more likely than decreases in biological activity. For example, guinea pig insulin has almost full biological potency but less than 10^{-3} times the immunochemical potency of other mamalian insulins in immunoassay systems employing antibodies developed in man or guinea pigs to beef or pork insulin (133). Insulins from fish and many lower vertebrates are even more unreactive in these systems (134).

With the scant amount of phylogenetic data available, we could not predict apriori the degree of cross immunoreactivity between invertebrate and vertebrate insulin-like peptides. However, it did appear that cross-immunological activity has been conserved in the insulin peptide family (3,70,76,82,83,85,86,88,98,105). Examination of available sequence data indicated that much of the structure of insulin was conserved during evolution. In fact, the human and porcine hormone are identical, while

the bovine molecule differs by only 2 amino acids. Hagfish insulin, the most primitive vertebrate hormone whose amino acid sequence has been determined (106), is approximately 80 percent conserved relative to the porcine hormone. Using the rate of mutational acceptance for insulin of 4 accepted point mutations per 100 residues per 100 million years and an estimate of 900 million years since the divergence of insects and mammals (63), it might be expected that insect and vertebrate insulins may differ by about 40 percent in sequence and that basic elements of their structures are similar. Therefore, it is perhaps not surprising that a heterogeneous population of antibodies for mammalian insulin did cross-react with the insect insulin-like peptide found in royal jelly, hemolymph, and other insect tissues (70,76,83,85,86,98).

Insects are not the only invertebrate class of animals that respond to treatment with vertebrate insulin. Vertebrate insulin caused effects such as glucose uptake, glycogen synthesis or hypoglycemia in the following invertebrate animals: a protozoan, Tetrahymena pyriformis (99); a planarian, Polycelis nigra (101); a cestodian, Taenia crassiceps (100); a gastropod mollusc, Strophocheilus oblongus (1); and some bivalve moluscs, Anodonta cygnea (3), Unio pictorum (3), and Meretrix casta (2). Insulin-like biological activity (e.g., hypoglycemia, glycogen deposition, and glucose oxidation) was observed when extracts from invertebrates were assayed in vertebrate

tissues (Table 2). The invertebrates tested include: the clam, Mya arenaria (4), starfish, Pisaster ochraceous (5) and Asterias rubens (103), snail, Helix pomatia (104) and Strophocheilus oblongus (105), crab, Callinectes sapidos (160) and Carcinus maenas (51), sea squirt, Ciona intertinalis (6), whelk, Buccinum undatum (6), scallop, Pectin maximus (6), octopus, Eledone cirrosa (6), and oyster, Ostrea edulis (L.) (7). Insulin-like immunoreactivity has been localized in gut tissue by radioimmunoassay or immunocytochemical analysis in the bivalve molluscs, Mytilus edulis (88), Anodonta cygnea (3), and Unio pictorum (3), and the snail, Strophocheilus oblongus (105).

The Physiological Function of Insulin-like Peptides in Invertebrates

Although there may be some exceptions (73,75,93), physiological functions of insulin in invertebrates generally parallel those in vertebrates. These include glucose uptake (96-101), hypoglycemia or hypotrehalosemia (1-3), glycogen synthesis (3), and triglyceride release (74). Concerning M. sexta, Tager et al. (70) demonstrated hypotrehalosemic activity of immunoreactive insulin from the adult neurosecretory gland complex. They suggested that these insulin-like peptides are secreted into the hemolymph where they probably act on peripheral tissues to promote sugar uptake and oxidation. The present study has confirmed

Table 2—Occurrence of insulin-like activity in invertebrates.

Species	Material	Assay	Quantity (ng/g)	Reference	Comments
Clam, *Mya arenaria*	Whole body	Blood sugar levels of the rabbit	(+)*	(4)	Tissue extracted according to method developed for isolation of insulin from ox pancreas
Starfish, *Pisaster ochraceous*	Pyloric caeca	Glycogen deposition in mouse tissue	288	(5)	An acid isopropanol extract was used for the assay. Insulin activity was neutralized by guinea pig antiserum to ox insulin.
Snail, *Helix pomatia*	Protein gland & hepatopancreas	Glucose oxidation epididymal in fat pad	(+)	(104)	Insulin activity neutralized by anti-sera to vertebrate insulin
Starfish, *Asterias rubens*	Pyloric caeca	Glycogen deposition in mouse tissue	(+)	(133)	
Sea Squirt, *Ciona intestinalis*	Digestive tract	"	228	(6)	An acid alcohol extract was used for the assay. Insulin activity was neutralized by antisera to vertebrate insulin.
Whelk, *Buccinum undatum*	Esophagus stomach intestine	"	728 476 168	"	"
Octopus, *Eledone cirrosa*	Intestine cecum	"	3840 64	"	"

Table 2—continued.

Table 2—continued.

Species	Material	Assay	Quantity (ng/g)	Reference	Comments
Scallop, *Pecten maximum*	Esophagus	Glycogen deposition in mouse tissue	396	(6)	An acid alcohol extract was used for the assay.
Crab, *Carcinus maenas*	Hepatopancreas	"	540	"	"
Lobster, *Homarus gammarus*	Digestive tract	"	(-)†	"	"
Jelly fish, *Aurelia aurita*	Gastric pouch & radial canal	"	(-)	"	"
Sea anemone, *Metridium senile*	Digestive tract	"	(-)	"	"
Oyster, *Ostrea edulis L.*	Hepatopancreas	Glycogen deposition in rat & oyster tissue	(+)	(7)	A gel filtered acid alcohol extract was used for the assay.
Snail, *Strophocheilus oblongus*	Hepatopancreas and gut	Radioimmunoassay	(+)	(135)	

*(+) = activity detected but not quantitated
†(-) = activity not detected

that such a peptide is present in hemolymph, but further study is required to determine how it functions physiologically. The insulin-like peptide may produce hypotrehalosemia by stimulating membrane transport of sugar or by inducing an enzyme that metabolizes trehalose.

The level of insulin-like peptides in M. sexta hemolymph determined by radioimmunoassay in this study was 5 ng insulin equivalents per ml. Actual levels may be higher because of the probable low immunological cross-reactivity of the antibody used. This concentration is comparable to vertebrate levels which can range from 0.2 to more than 8 ng/ml (123).

The function of the insulin-like peptides in royal jelly remains to be determined, especially with regard to whether or not it is indeed a hormone. Although the polypeptide has biological activity and affects carbohydrate metabolism in other animals (70,82), it has not been tested physiologically in the honeybee; and we do not yet know if it is released into the hemolymph for delivery to peripheral tissues. Royal jelly is a secretion of the hypopharyngeal glands of the honeybee adult and it is needed by the developing larvae and queen bee. Perhaps the insulin-like peptide plays a nutritional role related to the high carbohydrate content of royal jelly (37 percent of dry weight). The royal jelly insulin-like peptide may act to promote normal growth of honeybee larvae (insulin promotes differentiation and growth in both vertebrates and insects

(79,80,83)) or it may have no biological function other than being a protein food source. Unless the immunoreactive insulin in royal jelly is bound to a protective carrier such as lipid or another protein or is intrinsically resistant to hydrolysis by the digestive proteases of the honeybee, it is probably inactivated to small peptides and amino acids in the insect gut. Further investigation is needed to explain the physiological role of insulin in royal jelly.

Concluding Remarks

Fundamentally, it is not surprising that invertebrate regulatory mechanisms are similar to those of the vertebrates. We are now beginning to understand that insects and vertebrates do use similar peptide hormones as regulatory agents, as well as dissimilar ones (31). In Part I of this thesis, we have provided further evidence that insects use a peptide hormone similar to vertebrate insulin. Although the chemical structure of the immunoreactive insulin and the mechanism for its secretion and action remains to be determined, its immunological and biological similarities to the vertebrate counterpart suggests that this hormone arose remarkably early during animal evolution and has remained nearly constant and widespread.

PART II

SOME EFFECTS OF VERTEBRATE ORAL HYPOGLYCEMIC AGENTS IN INSECTS

INTRODUCTION

The discovery of physiologically active, insulin-like polypeptides in the neuroendocrine system of insects indicates that the invertebrate and vertebrate hypoglycemic factors are related (70,82-85) and that pharmacological agents which affect insulin action in vertebrates might function similarly in insects. Oral hypoglycemic agents such as phenformin, tolbutamide, and chlorpropamide lower vertebrate blood sugar levels. Because this research was a part of an overall study to determine how insects regulate carbohydrate metabolism, we wished to determine whether these drugs affect insects in a similar manner. If indeed they did, then certain similarities between the insect and vertebrate carbohydrate metabolism could be inferred.

LITERATURE REVIEW

Oral Hypoglycemic Agents

Despite long usage of oral hypoglycemic drugs in diabetes therapy, their exact mode of action in vertebrates is not entirely understood, and nothing is known about their effects in invertebrates. The following will summarize what is known about the actions of these drugs in vertebrates.

A. Classes of Oral Hypoglycemic Agents

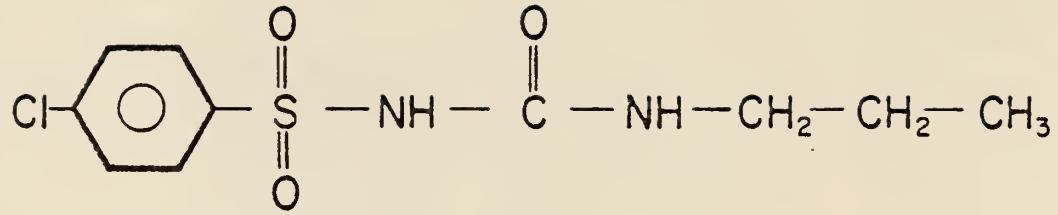
There are two major classes of oral hypoglycemic drugs, the sulfonylureas and the biguanides. The common structural components are $R_1\text{-}SO_2\text{-}NH\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH\text{-}R_2$ and $R_1\text{-}NH\text{-}\overset{NH}{\overset{\|}{C}}\text{-}NH\text{-}\overset{NH}{\overset{\|}{C}}\text{-}NH_2$ respectively where R_1 and R_2 are alkyl or aromatic substituents (141). Figure 8 shows the chemical structures of the three drugs used in this study: chlorpropamide, tolbutamide and phenformin.

B. Mode of Action

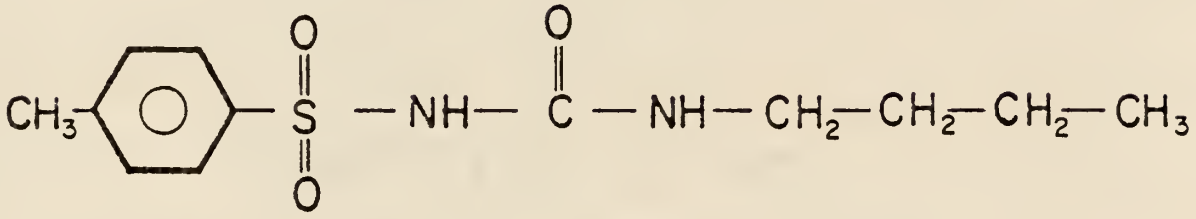
For sulfonylurea compounds to be effective, a functioning pancreas is required (150,151). The primary action of acute administration is increased insulin release, followed by decreased blood glucose levels (142). Chronic sulfonylurea therapy eventually promotes decreased insulin levels (143,144). The causes for these long term effects are not well understood, but they may be due to an enhanced glucose uptake by peripheral tissues, a decreased glucose

Figure 8. Hypoglycemic drugs. A. Chlorpropamide; B. Tolbutamide; C. Phenformin

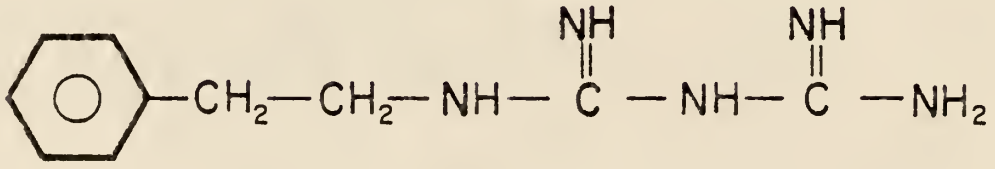
A.



B.



C.



output by liver (146-148), an increased number of binding sites for insulin in target tissues (149), or a depletion of insulin in the pancreas. Sulfonylureas are effective as hypoglycemic agents in both the diabetic and nondiabetic animal.

Biguanides such as phenformin are believed to lower blood sugar levels by disrupting oxidative metabolism of carbohydrates (accelerating anaerobic glycolysis) (152). They have also been found to decrease glucose absorption from gastrointestinal tract (125,153) and to inhibit hepatic gluconeogenesis (125). Different species of animals show varying hypoglycemic responses (108). Phenformin is an extremely effective hypoglycemic agent in the monkey, guinea pig, and diabetic human and rat (109).

Phenformin is the most active oral hypoglycemic agent utilized in this study. It is followed by chlorpropamide. Of the sulfonylureas, chlorpropamide is more active than tolbutamide, probably because it is more slowly metabolized (154).

Side effects of the hypoglycemic drugs are most pronounced with biguanide derivatives. The most common effects observed with phenformin include tissue hypoxia, lactic acidosis, anorexia, nausea, metallic taste, and gastrointestinal disorder (125,154-156,159). Over doses of either the biguanide or sulfonylurea drugs eventually lead to hypoglycemic coma and death.

MATERIALS AND METHODS

Hypoglycemic Drugs

Phenformin HCl [N-(2-phenylethyl)imidodicarbonimidic diamide monohydrochloride], chlorpropamide [4-chloro-N-(propylamino)carbonyl benzenesulfonamide] and tolbutamide [N-(butylamino)carbonyl-4-methyl- benzenesulfonamide] were obtained from Ciba-Geigy Corporation, Ardsley, NY, Pfizer Pharmaceuticals, Inc., Barceloneta, P.R. and Upjohn Co., Kalamazoo, MI., respectively.

Insect Rearing Studies

A. Indian Meal Moth

Plodia interpunctella, Indian meal moth eggs were obtained from Dr. W. H. McGaughey, Research Entomologist, U. S. Grain Marketing Research Center, AR, SER, USDA, Manhattan, Kansas, and were collected according to the method of R. Kinsinger (113).

The diet for the Indian meal moth, *Plodia interpunctella*, was made according to Kinsinger (113). It consisted of 500 g coarse ground wheat, 500 g wheat shorts, 50 g wheat germ, 40 g Brewers yeast, 2 g sorbic acid and 2 g methyl p-hydroxy benzoate. The latter two components were added to prevent fungal growth. The dry ingredients were thoroughly hand mixed before incorporating a mixture of 120 ml honey, 120 ml glycerin, and 60 ml distilled water.

Phenformin was mixed with the diet in concentrations ranging from 500 to 10,000 ppm. An appropriate number of phenformin tablets were pulverized with mortar and pestle, suspended in 10 ml deionized water and mixed thoroughly with 100 g of diet.

Seventy-five Indian meal moth eggs were deposited on top of each 100 g of treated diet in a 0.5 l jar. Filter paper was used as an air permeable lid for individual jars and was held in place by a Mason jar ring. Feeding tests were performed in duplicate at 27°C and 80 percent relative humidity. The light cycle was 12 hours of light followed by 12 hours of dark. Adult insects were collected and counted as they emerged. Approximately 30 days were required for the development from egg to adult.

B. Tobacco Hornworm

Manduca sexta, tobacco hornworm eggs were obtained from Dr. R. A. Reinecke, Research Entomologist, Metabolism and Radiation Laboratory, AR, SEA, USDA, Fargo, North Dakota.

The method used to prepare diet for the tobacco hornworm is the same as that reported by Bell and Joachim (114). Diet was made by combining 240 g wheat germ, 108 g casein, 96 g sucrose, 36 g Wessons salt mixture (Nutritional Biochemical Corporation, Cleveland, Ohio), 48 g Brewers yeast, 1 g cholesterol, 6 g sorbic acid, 3 g methyl p-hydroxybenzoate and 0.5 g streptomycin. Then 60 g fine ground agar was added to three liters of boiling water. The

agar solution was allowed to boil for 2 or 3 minutes before pouring it into a 1.5 gallon waring blender. The dry ingredients were added while the blender was slowly rotating. The mixture was thoroughly blended at high speed before placing the blender container in cold water bath. The medium was cooled to 24°C before replacing the blender container on its base and adding 12 g ascorbic acid, 30 ml vitamin solution (a mixture of 150 mg nicotinic acid, 75 mg riboflavin, 35 mg thiamine, 35 mg pyridoxine, 35 mg folic acid, 3 mg biotin, and 150 ml distilled water), 12 ml linseed oil, and 60 ml of 4 percent formalin. The diet was then mixed thoroughly for 2 minutes before pouring the contents into a container.

The concentrations of phenformin in the agar based diet ranged from 500 to 10,000 ppm; the concentrations of chlorpropamide ranged from 4,500 to 10,000 ppm, and tolbutamide was tested at only the 10,000 ppm level. The three hypoglycemic agents were thoroughly mixed with the partially cooled liquid agar medium which was then allowed to solidify.

Feeding tests were conducted at 27°C and about 50 percent humidity in a Scherer, controlled light and temperature growth chamber. The light cycle was 16 hours of light followed by 8 hours of dark. Under these conditions, approximately 33 days were required for the cycle from egg to adult moth to be completed. After the eggs hatched, the required number of larvae were left on the media while other

larvae and any unhatched eggs were removed. The larvae were weighed every two to four days. After the second stadium, it was necessary to separate the larvae from each other to avoid cannibalism. The larvae were observed closely at the prepupae stage in order to determine whether they voided their gut contents. Mold growth was likely unless the rearing cups were cleaned. Placing a piece of filter paper in the rearing cups was also helpful in absorbing moisture and in preventing microbiological contamination.

C. Confused Flour Beetle

Tribolium confusum, confused flour beetle eggs, were obtained from the laboratory culture maintained at the U. S. Grain Marketing and Research Laboratory, Manhattan, Kansas.

The growth medium for the confused flour beetle, Tribolium confusum, was either coarsely ground whole wheat or commercially purchased enriched white flour containing ca. 3 percent Brewers yeast. Pulverized phenformin tablets were added to the treatments to produce concentrations ranging from 650 to 20,000 ppm phenformin.

Fifty confused flour beetle eggs were deposited on top of 50 gm of treated diet in 5 cm deep by 8.5 cm wide clear plastic cups. A loosely fitting clear plastic lid was used to cover each cup. When the adults emerged, it was necessary to tape the container closed to prevent their escape. Feeding tests were performed in duplicate and incubated at 27°C and 50 percent humidity in a Forma walk-in

constant temperature and humidity chamber. The light cycle was 12 hours of light followed by 12 hours of darkness. Under these conditions, approximately 42 days were required to complete the life cycle.

After the eggs hatched, a representative sampling of the insects in each test were weighed at two day intervals as follows: a fine sable hair brush was used to selectively transfer 10 individuals from the rearing cups to tared weighing paper. Care was taken to avoid transferring diet to the paper. The insects were then weighed using a Mettler H54 balance.

Dose-Mortality Relationships

The LC 50 is an estimate of the concentration of dietary chemical that would kill 50 percent of a given population. Data for this calculation was obtained by dividing test animals into groups of moderate size and giving each group one of a series of increasing concentration of chemical. Mortality percentages were calculated from the difference between the number of eggs or larvae added and the adults that emerged. Values were corrected for mortality in untreated samples. The corrected mortality values were then used to establish the ppm of chemical per weight of diet required to suppress 50 percent of the control population (LC 50). Where statistically possible, toxicity data was subjected to a 3 step probit

analysis using a Wang[R] 700 program (135,136) in order to accurately determine the LC 50.

Effect of Chlorpropamide on Hemolymph Trehalose Levels and Fat Body Glycogen in Tobacco Hornworm

A. Collection of Hemolymph and Fat Body from Manduca sexta

M. sexta larvae weighing 1.0 ± 0.2 g were placed on a diet containing 0.0 or 10,000 ppm chlorpropamide. The larvae were allowed to feed (ca one day) until they weighed 2.2 ± 0.3 g. The animals were weighed and then fasted for at least one hour before cooling to 4°C, collecting the hemolymph, and dissecting out the fat body (70,137). Both hemolymph and fat body were quick frozen for storage. The fat body was placed in a small vial before freezing and the hemolymph was collected by bleeding each larva into individual test tubes containing a few crystals of 1-phenyl 2-thiourea (PTU) a phenol oxidase and trehalase inhibitor (115).

B. Purification and Quantitation of Hemolymph Trehalose

1. Saccharide Chromatography

Hemolymph trehalose was purified using the method of Kramer et al. (117). Bio-Gel P-2 100 to 200 mesh porous polyacrylamide beads were used to prepare a 0.9 by 120 cm column. The fractionation range of the Bio-Gel P-2 column was 100 to 1,800 daltons. Trehalose (molecular weight of 342) is separated from glucose (molecular weight of 180) on

this column. Methods used to prepare the column and apply sample were similar to those described on page 34, except that ca 27 g Bio-Gel P-2 and 200 ml of the column buffer, 20 mM Tris, 0.1 M NaCl buffer pH 7.3 were used in the gel hydration step. The elution volumes of trehalose and glucose from the P-2 column were determined by applying a mixture of 100 ul each of 200 mM trehalose and 100 ul of 200 mM glucose to the column, eluting with the 0.02 M Tris, 0.1 M NaCl buffer, and analyzing successive 1.1 ml fractions of the effluent for the sugars using the anthrone assay.

2. Anthrone Assay for Trehalose and Glucose

The anthrone reaction (116) was utilized to determine neutral hexoses such as glucose and trehalose. In this reaction furfural derivatives are formed in concentrated sulfuric acid which reacts with the yellow anthrone reagent to produce a blue green color. The anthrone reagent was made by adding 720 ml of concentrated sulfuric acid to 280 ml water cooled in an ice bath. While the mixture was still warm, 500 mg anthrone was added, allowed to stand overnight and then thoroughly mixed.

The reaction was carried out in 13 by 100 mm pyrex tubes containing 100 ul of the sample to be assayed. A repipet was used to quickly add 1 ml anthrone reagent. Then the tubes were vortexed, capped with a glass marble, heated in a boiling water bath for 15 minutes and immediately cooled to room temperature in a water bath before

determining the absorbance of the solution at 620 nm on a Beckman DU or a Cary 118C spectrophotomer. The amount of sugar present in the hemolymph was quantitated by comparing absorbance values with those obtained with 2 mM trehalose and 2 mM glucose standards and using Beer's law (138). Tubes with column buffer replacing the sample volume were used as controls. Before any anthrone assays were performed on unknown samples, ca 20 trehalose and glucose standards were analyzed and an average standard absorbance was determined. Each time an assay was performed, at least three new standards were also analyzed and normalized to previously determined standards.

C. Glycogen Level in *Manduca sexta* Fat Body

Glycogen determinations were made according to a modification of the method of Carroll *et al.* (118). After lyophilization of fat bodies, 4 to 6 mg of tissue were mixed with 1 ml 30 percent KOH in a 5 ml pyrex centrifuge tube. The tube was then placed in boiling water for 15 to 30 minutes or until the tissue dissolved, cooled to room temperature and 15 mg Na_2SO_4 added. Subsequently, 2 ml absolute ethanol was added to precipitate the glycogen, the tubes vortexed and allowed to stand overnight at $4^{o}C$ before centrifugation in the cold. The supernatant was decanted and the precipitate redissolved in 1 ml of 0.5 percent KOH after which the sample was again treated with 2 ml ethanol, vortexed, incubated in the cold, centrifuged, and the

supernatants discarded. The resulting precipitate was redissolved in 1 ml 72 percent sulfuric acid and a 100 ul aliquot was subjected to the anthrone assay for glucose.

RESULTS

The Toxicity of Chlorpropamide and Tolbutamide to Manduca sexta

In order to determine physiologically active doses of the sulfonylurea compounds in insects, we first conducted a dose mortality study using the tobacco hornworm. An oral feeding test was performed that tested levels up to 10,000 ppm of the drugs admixed with the diet. Chlorpropamide was found to be the most toxic sulfonylurea. The chlorpropamide LC 50 for M. sexta was ca 8.5×10^3 ppm or about 3.4 mM in the diet (Table 3). Tolbutamide was less toxic and produced ca 30 percent mortality when present at 10^4 ppm in the diet.

The Physiological Effect of Chlorpropamide in Manduca sexta

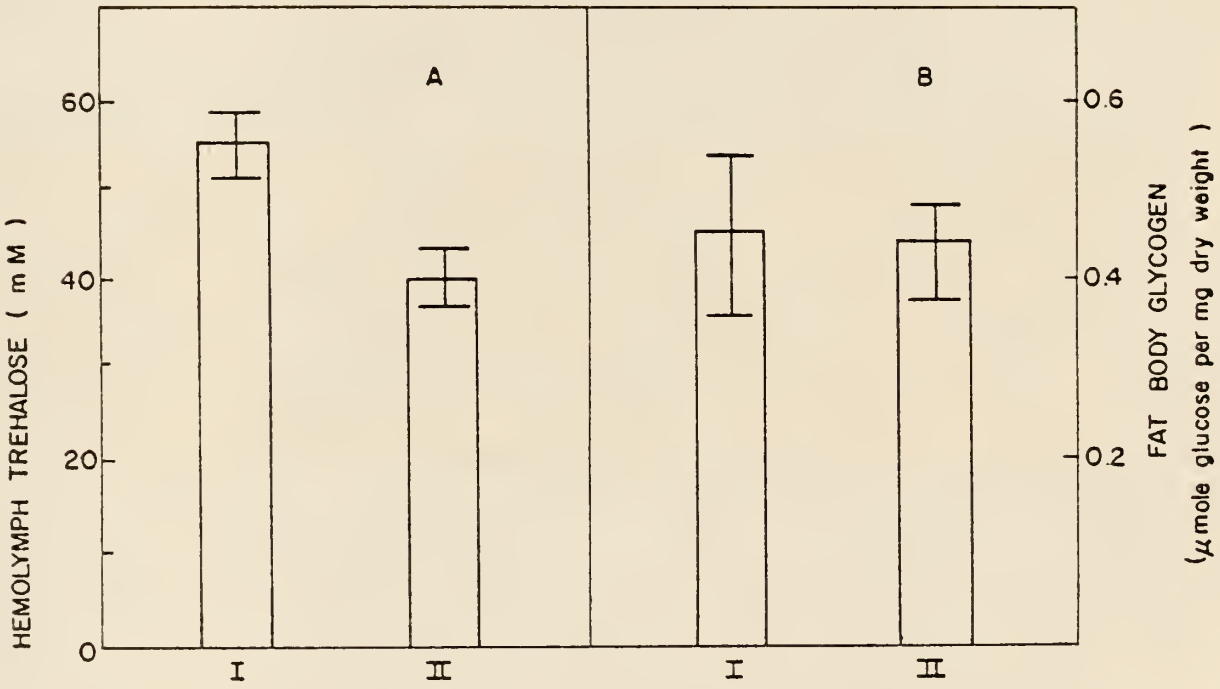
In order to determine whether chlorpropamide decreases blood sugar in invertebrates as it does in vertebrates, we fed the drug to last stadium larvae at approximately the LC 50 level and determined hemolymph trehalose and fat body glycogen levels. Trehalose fell by 23 percent in the drug treated animal from 54 ± 4 to 42 ± 2 mM (Figure 9). No effect was observed on fat body glycogen which remained at about 450 umole glucose per gram of dessicated tissue. Thus, chlorpropamide, a drug that induces insulin secretion and hypoglycemia in vertebrates acted as a hypotrehalosemic

Table 3. Diet levels of hypoglycemic drugs which suppress 50 percent of the insect population.

Drug	LC 50 (ppm x 10^{-3})†
*Manduca sexta**	
Chlorpropamide	8.5
Tolbutamide	>10
Phenformin	1.1 (0.8-1.3)
*Plodia interpunctella*δ	
Phenformin	3.3 (1.8-6.1)
Tribolium confusum¶	
Phenformin	13 (10-19)

*5-20 neonate larvae tested per dose. Run in duplicate.
†95 percent confidence limits given in parenthesis.
δ75 eggs added to medium treated homogeneously with phenformin. Run in duplicate.
¶50 eggs added to medium treated homogeneously with phenformin. Run in duplicate.

Figure 9. Effect of chlorpropamide on hemolymph trehalose and fat body glycogen of M. sexta. Mean values ± S.E.M. from eight experimental animals are shown. Fourth instar larvae (1.0 ± 0.2 g) were placed on normal diet and diet containing about 10^4 ppm chlorpropamide. Animals weighing 2.2 ± 0.3 g were sacrificed 1-2 days later. (A)--Hemolymph trehalose content from control hornworms (I) and hornworms fed diet supplemented with chlorpropamide (II). (B)--Fat body glycogen content from control (I) and chlorpropamide-treated (II) hornworms.


A
B
60
40
20
0
0.6
0.4
0.2
I
II
I
II
HEMOLYMPH TREHALOSE (m M)
FAT BODY GLYCOGEN
(μ mole glucose per mg dry weight)

agent in insects.

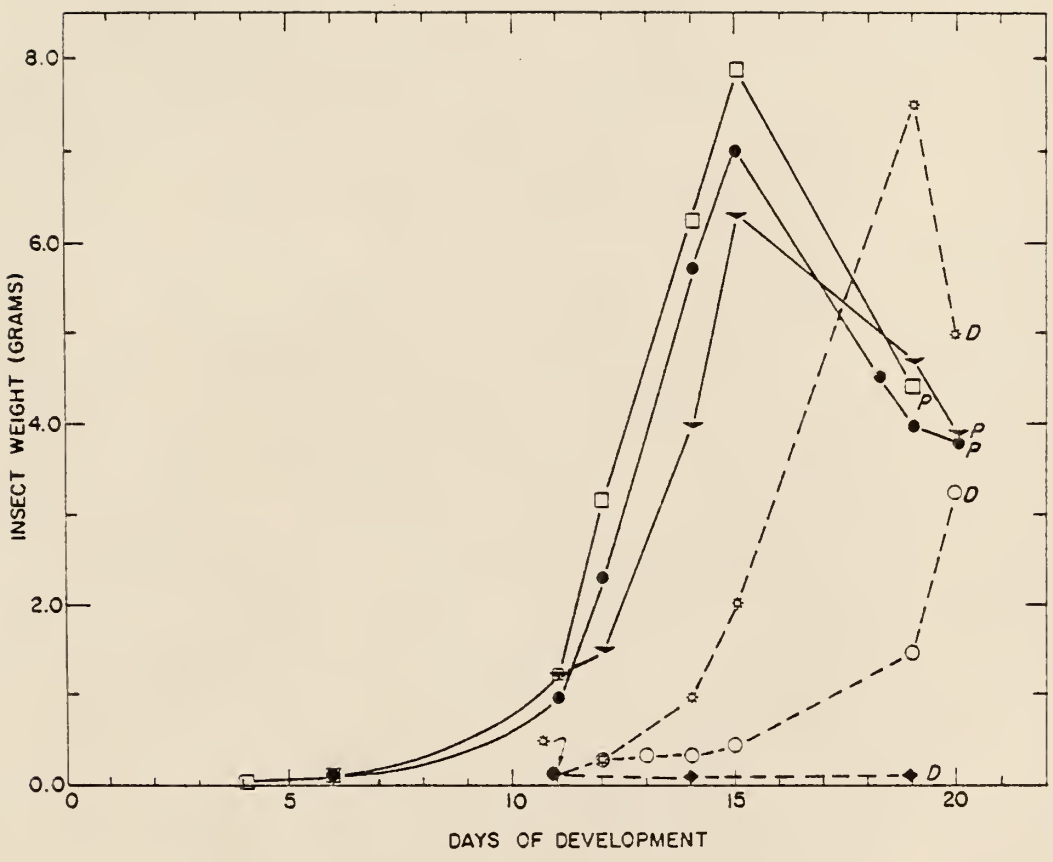
The Toxicity of Phenformin to Manduca sexta, Plodia interpunctella, and Tribolium confusum

Like the sulfonylurea drugs, the presence of phenformin in the diet was detrimental to insect development, but phenformin was about four times more toxic. The LC 50 for the tobacco hornworm was 1.35×10^3 ppm (Table 3), while that for chlorpropamide was only 8.5×10^3 ppm. Generally, as the amount of the phenformin in the diet was increased, greater numbers of larvae expired and the time required for pupation of surviving animals increased. Under standard conditions, larvae grow for about 19 days until they undergo pupal ecdysis (Figure 10). At $0.5\text{-}1.0 \times 10^3$ ppm phenformin, pupal development took one or two days longer and at $2.5\text{-}3.0 \times 10^3$ ppm, none of the larvae were able to pupate.

The Indian meal moth showed a response to phenformin much like the tobacco hornworm, except that Plodia was about three times more tolerant with an LC 50 = 3.3×10^3 ppm. It should be noted that this comparison may not be a reliable one since the diets used for each species were quite different in their physical make-up. The hornworm diet was more homogeneous and it might be expected to be more effective than the Indian meal moth diet in presenting a uniform dose of drug to the consumer.

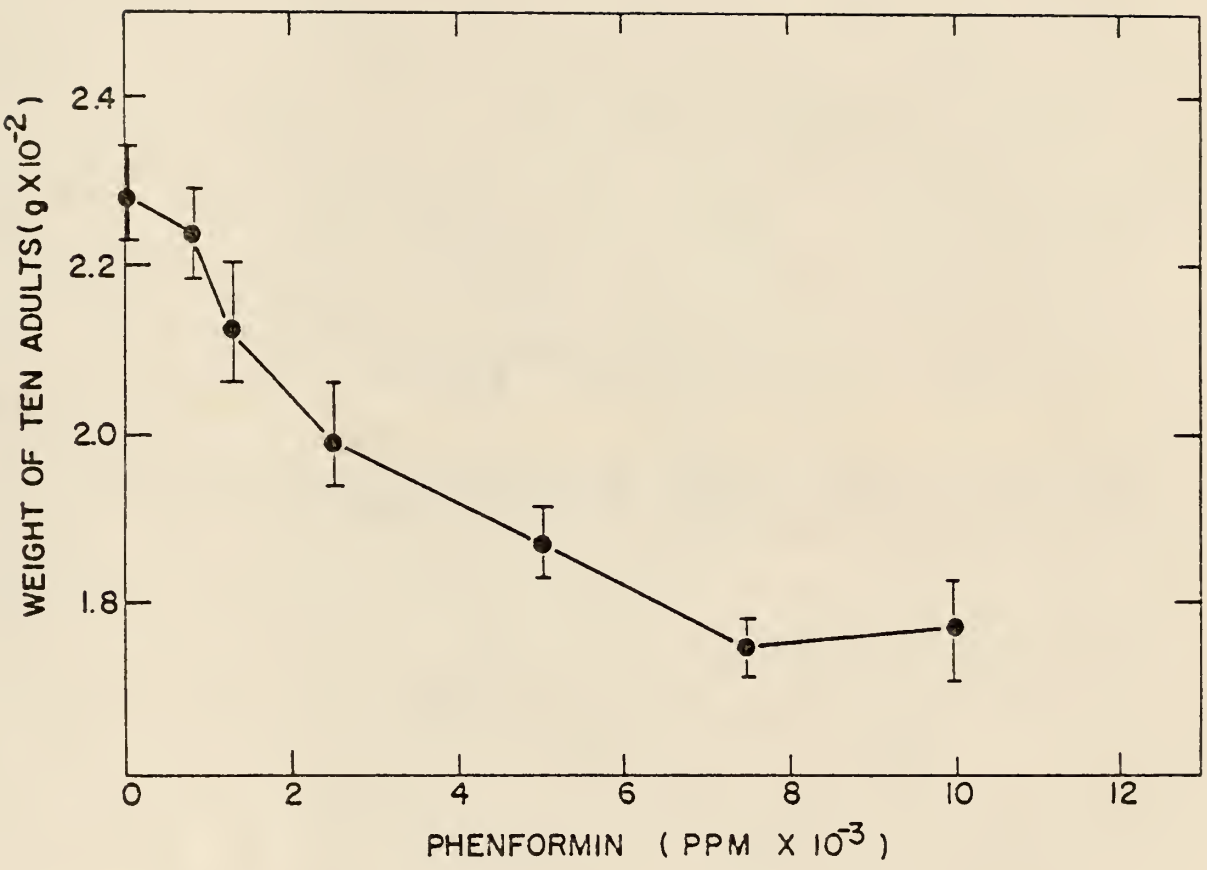
The confused flour beetle, Tribolium confusum, was the species found to be least susceptible to phenformin in this

Figure 10. Growth curves of M. sexta larvae fed standard diet (-□-) and diets supplemented with phenformin: 500 (-●-), 1 x 10^3 (-▼-), 2.5 x 10^3 (-✡-), 3 x 10^3 (-○-), and 5 x 10^3 (-◆-) ppm. D = death, P = pupation.


8.0
6.0
4.0
2.0
0.0
INSECT WEIGHT (GRAMS)
0
5
10
15
20
DAYS OF DEVELOPMENT
D
P
P
P
D
D

study. The LC 50 (1.3×10^4 ppm) was about 10 times greater than the hornworm's when the drug was admixed with the enriched flour diet (Table 3). Again this comparison must be interpreted cautiously because of the very different nature of the two diets used. The concentration of phenformin in the diet also affected the duration of the Tribolium life cycle. At 2×10^4 ppm, only 34 percent of the beetles developed to adults in ca 60 days whereas untreated adults emerged in 40 days. A weight reduction also occurred with the adult insects which survived the phenformin treatment (Figure 11). For example beetles grew to only 80 percent of their normal weight when fed $8\text{-}10 \times 10^3$ ppm phenformin. Accompanying abnormalities were also noted in some of the beetles such as cuticle discoloration and wing malformation. These observations suggest that phenformin interferes with tanning chemistry and imaginal disk development.

Figure 11. Effect of phenformin on the weight of the confused flour beetle, Tribolium confusum.


WEIGHT OF TEN ADULTS(g X 10^{-2})
2.4
2.2
2.0
1.8
0
2
4
6
8
10
12
PHENFORMIN (PPM X 10^{-3})

DISCUSSION

Sulfonylureas: Chlorpropamide and Tolbutamide

The results of this investigation suggest that sulfonylurea drugs used in diabetes therapy have similar actions in insects and vertebrates. When we examined the effects of the sulfonylureas in insects, we found that administering chlorpropamide and tolbutamide to the tobacco hormworm inhibited development with the former drug being the more effective. In vertebrates, chlorpropamide is the more active hypoglycemic agent and presumably is also the more toxic drug (125). Our investigation also demonstrated that chlorpropamide depressed the blood carbohydrate level in the tobacco hornworm, producing a hypotrehalosemic effect that is similar to the hypoglycemia observed in vertebrates after sulfonylurea administration. The physiological effect of tolbutamide was not determined.

The acute action of sulfonylurea drugs in vertebrates is beta-cell degranulation and insulin release from the pancreas (125). We propose that similar mechanisms operate in insects. Insulin-producing cells in insects analogous to pancreatic beta-cells may be stimulated by sulfonylurea agents to release insulin and produce a hypotrehalosemic state.

The other insulin effect that was looked for in insects upon administration of chlorpropamide was an increase in fat body glycogen levels. Such a response was anticipated

because insulin has this effect in vertebrate tissues. However no increases in glycogen levels were observed. Observations similar to ours were reported by Tager et al. (70) who injected M. sexta larvae with immunoreactive insulin from insect neurosecretory tissue. These injections only decreased blood sugar and did not increase glycogen levels in fat body. The overriding effect of chlorpropamide treatment may have been increased trehalose utilization and oxidation by peripheral tissues.

This study provided suggestive evidence for the presence of cells in insects that produce insulin. It is interesting to note that alloxan and streptozotocin, drugs used experimentally to selectively destroy pancreatic beta-cells in vertebrates and cause hyperglycemia (diabetes) and death, have physiological and toxic activities in insects. Ishay (110) found that feeding alloxan to Vespa orientalis larvae led to a marked increase in salivary glucose levels and caused death in a few days. Kramer et al. (126) found alloxan and streptozotocin to be lethal to M. sexta larvae at levels similar to effective doses for vertebrates (50-200 mg/kg body weight). In a histological study, alloxan injection caused cells in the corpus cardiacum, a neurosecretory organ which apparently secretes insulin in insects, to degenerate (Speirs, R. D. and Kramer, K. J. unpublished). All of these studies support the hypothesis that insects contain insulin producing cells in the neuroendocrine system and that the insulin-like peptide

is the hypotrehalosemic hormone.

Biguanides: Phenformin

We have less information on phenformin action in insects than we have on sulfonylurea action. All we know is that phenformin was at least 8 times more toxic than the sulfonylurea agents to insects. This trend is similar to that observed when vertebrate hypoglycemic activities of these two kinds of drugs are compared. In vertebrates phenformin is usually about 2 times more effective than chlorpropamide (125). This comparison of drug action is not a straightforward one since vertebrates take these drugs in carefully measured doses while the insects were fed the drug ad libitum admixed with diet.

The toxicity of phenformin to insects may result from an action on carbohydrate metabolism similar to that observed in vertebrates. The demonstration of hypotrehalosemia following phenformin administration would provide direct evidence for such an action.

The toxicity of phenformin was dependent on the species of insect tested. The confused flour beetle was four and twelve times less susceptible to the drug than the Indian meal moth and tobacco hornworm, respectively, This suggests that beetle species may be more resistant to the biguanide drug than are moth species. It was also evident that the two stored product insects, Plodia and Tribolium, were more

tolerant of phenformin than the phytophagus insect, Manduca. In general stored product insects are found to be more hardy than are insects with a dietary requirement for fresh plants (138).

One other factor must be considered when making toxicological comparisons such as we have done, that is, the type of diet used. Each of our experimental animals were fed on a different type of diet. Manduca fed on a semiartificial agar based diet that was relatively homogeneous. Tribolium fed on a coarsely ground wheat medium that was very heterogeneous. We found that diet consistency probably plays a negligible role in determining toxicity levels since phenformin had the same toxicity (within experimental error) to Tribolium in both the ground wheat and flour diets. If heterogeneity was important, then phenformin might have been expected to be more toxic to Tribolium in flour than in ground wheat.

Concluding Remarks

The stresses caused by the sulfonylureas in insects could very well be hypotrehalosemic hormone (insulin)-mediated responses. Just as in vertebrates, the sulfonylurea compounds may increase insulin output in insects with resultant lowered blood sugar and death at high doses. Our results in both Part I and Part II of this thesis, together with those of others, support the

hypothesis that insects have the potential to suffer from pathological states due to insulin over or under production. In view of these conclusions, it is interesting to note that the hyperglycemia associated with cautery of the medial neurosecretory cells of the Calliphora brain results in manifestations (i.e., polydipsia) similar to conditions of vertebrate insulin underproduction, diabetes (30).

The insecticidal action of the biguanide derivative may result from actions on carbohydrate metabolism similar to that observed in vertebrates, i.e., increased glycolysis, decreased gluconeogenesis, and decreased glucose absorption. However, to fully determine whether the true pharmacological action of either the sulfonylureas or the biguanides is as stated above, more detailed studies such as biochemical, morphological, histological and immunocytochemical analyses of their effects are needed. As has previously been done with vertebrates, it should be determined whether hyperinsulinemia also occurs in our invertebrate animals under the influence of sulfonylurea administration. If indeed these vertebrate agents prove to kill insects by disrupting carbohydrate metabolism, then it might be worthwhile to develop a screening procedure to search for chemicals that are active in pest insects and not in vertebrates. Such compounds may have potential for use as pesticides.

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INSECT CARBOHYDRATE METABOLISM: PARTIAL PURIFICATION OF INSULIN-LIKE PEPTIDES AND SOME EFFECTS OF VERTEBRATE HYPOGLYCEMIC AGENTS IN INSECTS

by

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This research was part of an overall study to determine how insects regulate carbohydrate metabolism. In the first part of this thesis, the partial purification of hypotrehalosemic, insulin-like factors from hemolymph of the tobacco hornworm, Manduca sexta and from royal jelly of the honeybee, Apis mellifera, is described. An insulin-like peptide from the hornworm was partially purified by acid extraction of lyophilized hemolymph and subsequent affinity chromatography on agarose coupled to anti-insulin immunoglobulin. Honeybee immunoreactive insulin was partially purified by acid extraction of lyophilized royal jelly, delipidatation, gel filtration, and affinity chromatography. Two different royal jelly insulin-like peptides were detected. One peptide had a rather high molecular weight ($\geq 10^4$) and the other was approximately the size of bovine insulin (5×10^3). The quantities of insulin-like peptides present in hemolymph and royal jelly were determined using insulin radioimmunoassay. The yield of immunoreactive insulin from M. sexta hemolymph was 1×10^{-7} gram porcine insulin equivalents per gram of lyophilized hemolymph or 5×10^{-9} gram per ml of native hemolymph. The concentration of immunoreactive insulin in hemolymph was 10^{-9} M. The yield of immunoreactive insulin from royal jelly was 1.4 and 1.5×10^{-9} gram per gram of native royal jelly for the high and low molecular weight forms, respectively.

The second part of this thesis was concerned with the

effect of vertebrate hypoglycemic agents in insects. The oral toxicity (LC 50) of chlorpropamide, tolbutamide and phenformin for the tobacco hornworm was 8.5×10^3, 10^4, and 1.1×10^3 ppm in diet, respectively. Chlorpropamide at 10^4 ppm in the diet lowered blood sugar levels by 23 percent, but did not change fat body glycogen levels. Phenformin was toxic to the Indian meal moth and confused flour beetle with LC 50 values of 3.3×10^3 and 13×10^3, respectively. Phenformin also delayed maturation and caused cuticle discoloration and malformation in the latter species. The confused flour beetle was more resistant to phenformin than the two moth species while the two stored product insects (Indian meal moth and confused flour beetle), were more tolerant of the drug than the phytophagus insect (tobacco hornworm).